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14. ABSTRACT

This study has rested on two basic hypotheses. The first is that agents given during and (for a short period of time) after resuscitation can favorably influence the response to hemorrhagic shock and injury. The second is that studies of gene response studies, specifically microarray and RT-PCR studies, could be used to assess the response of patients being treated for injuries to such therapeutic agents. To date, both hypotheses have been shown to be true in the animal model. Three therapeutic agents have been identified, of which glutamine appears to be the most suitable for initial use in patients. Initial clinical studies have been carried out to validate these hypotheses in patients, by using glutamine in patients immediately following resuscitation.

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INTRODUCTION

Shock is a leading cause of death among American soldiers wounded in battle. Unless an injury is immediately lethal, most deaths result from hemorrhagic shock or from its late sequelae, septic shock and multiple organ failure. There is a critical time in shock, which appears to be the period during which the patient is being resuscitated. Resuscitation is associated with a massive activation of the inflammatory reaction, producing immunosuppression, and rendering the patient vulnerable to sepsis and its sequelae. The *goal* of this research program is to develop new treatments for hemorrhagic shock to be administered before or during initial resuscitation. These treatments will be applied by front-line responders on the battlefield (and first responders in civilian life) as well as by fixed facilities, such as a Forward Surgical Team or a Combat Support Hospital. Suitable agents must be non-toxic and have a very broad therapeutic ratio, so that they can be given safely to injured soldiers, and must be easy to administer under combat conditions. In previous work, allopurinol, a xanthine oxidase inhibitor, was found beneficial in a shock model (1). In other work, using an animal model of hemorrhagic shock (2), glutamine, glutamine-alanine dipeptide, and arginine have shown efficacy. (3,4) Studies with crocetin have also shown efficacy (5), but this agent is not available in a form which can be administered to patients. Others have advocated the use of DHEA, but studies in our laboratories have failed to show a useful effect (6,7). Studies with omega-3 fatty acids have shown promise, but are still at an early stage.(8) Current research under this contract has focused on glutamine and arginine.

The *method* of the research is to measure the response of the genome to hemorrhagic shock using microarray studies. The effect of these agents in patients will be compared to their effect in the animal model, to assess whether they will be effective in the clinical setting.

BODY

We have proposed to develop a pharmacologic strategy, with the aim of reducing the intensity of the post-shock and post-injury inflammatory response. This strategy should be based on one (or more) agent(s) that are safe and can be given early in resuscitation, even by first responders and before resuscitation has begun.

The identification of such agents, which can be given at the beginning of resuscitation, or even before fluid resuscitation is begun, has been the subject of a great deal of research. In our own laboratories, during previous studies using several experimental animal models of hemorrhagic shock, we have identified several compounds that have shown therapeutic effectiveness, specifically crocetin, glutamine, and glutamine-alanine dipeptide. (1-9) There is convincing experimental evidence that these agents enhance cellular recovery following shock, and, importantly, that they significantly improve survival. These agents all appear to have two beneficial effects. First, they appear to reduce mitochondrial damage, which is perhaps the mechanism for

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improved recovery of tissue ATP levels. Reduced mitochondrial damage has been shown by decreased levels of cytoplasmic cytochrome c and by decreased activity of caspases, notably caspase 3 and 7, and by decreased apoptosis. Second, they appear to suppress the cytokine response, at least in part by inhibiting the activation of the transcription factor NF-κB. The activation of NF-κB is dependent among other factors on the proteasome, a large cellular proteolytic complex that has a regulatory role in the inflammatory process. In parallel investigations, agents that inhibit proteasome activity have also been shown to suppress cytokine production in response to inflammatory stimuli. (10-18)   Previous and ongoing research has established similarities between cytokine release induced by sepsis and that induced by hemorrhagic shock. It is becoming increasingly apparent that these similarities may reflect similar changes in the proteasome, a line of research which has been pursued by our laboratories, and which touches on the present investigation in significant aspects.

KEY RESEARCH ACCOMPLISHMENTS

I. Research objective #1: Establish and validate the micro-array studies, using the animal model. *Deliverable*: A panel of genes that are reproducibly altered in white blood cells and in liver and muscle by shock and resuscitation**.**

1. These preliminary studies were carried out, and presentation and/or publications are being prepared. (See list of publications, below).   Collaboration for these studies was with the laboratory of Dr. Peter Smith, Kansas University Medical Center, Kansas City, KS. This facility carried out micro-array analysis of both animal and human genomes.
2. To summarize the work in this area, the four experimental groups noted below were studied. As shown in the accompanying table, genomic response was quite marked even in the control and shock groups. The microarray analysis used 31,099 probes, from which about 12,500. These data indicate that even the sham surgical procedure was a strong stimulant to the gene response in white blood cells. The overall pattern of the response to untreated shock and to shock with either form of resuscitation was qualitatively similar. This data supports previous work by many others indicating that so-called hemorrhagic shock procedures are, in fact, a combination of trauma and shock. As noted below, it is quite feasible to identify genes that are differentially responsive to sham, shock, and shock with resuscitation. Please refer to the pathway analysis, below.

Experimental group	Genes upregulated	Genes downregulated
Sham at 30 minutes vs sham baseline	4993	7178
Shock at 30 minutes vs shock baseline	4758	7579
Shock + LR at 30 minutes vs shock + LR baseline	5554	7510
Shock + LR + arginine at 30 minutes vs baseline	6143	6325

3. A list of signature genes has been defined. The methodology for identifying these genes was as follows. All of the gene responses were uploaded into a web-based system maintained by Ingenuity Pathways Analysis (Ingenuity Systems,

; see also below, #8). This system combines data analysis with an extensive genome knowledge base. Genes that showed a strong up-regulation or down-regulation (at least fivefold) to shock, as compared with sham controls, were selected. They were considered as possible signature genes if they showed a difference in their response to shock and to one of the two treatment arms, either fluid resuscitation (shock + LR) or fluid resuscitation plus arginine (shock + arginine). The genes meeting these criteria numbered 390. Selection among these was then carried out by eliminating genes that appeared to be irrelevant to the inflammatory reaction, such as oncogenes. Some genes were selected because they appeared to be related to the known components of the inflammatory reaction (cytokines).   Several genes were placed on the list because their response in this shock model suggested that they would be good markers of the response to the surgical preparation and to shock (B3GALNT, F3, GRID2, GRM5).   The final list of 20 "signature" genes (below) are to be considered representative of genes which react strongly to the shock preparation, and which appear to be modified by treatment. While they are not the only such genes, they are a usable list of genes to be looked at in future investigations.

Signature Genes

Gene	*Protein and function*
ALOX5	Arachidonate 5-lipoxygenase (cytoplasmic enzyme)
B3GALNT1	Beta 1,3-N-acetyl/galactosaminyltransferase 2, membrane-bound galactotransferase enzyme
CXCL3	Chemokine (C-X-C motif) ligand 3, chemokine, cytokine
CXCL11	Chemokine (C-X-C motif) ligand 11, chemokine, cytokine
F3	Coagulation factor III, membrane receptor
GRID2	Glutamate receptor, ionotrophic, delta 2 (ion channel)
GRM5	Glutamate membrane receptor
HSPA1B	Heat shock 70kDa protein 1b
HSPBAP	HSPB (heat shock 27kDa) associated protein 1, binds HSPB1
IL1A	Interleukin-1, alpha, cytokine
IL1R2	Interleukin 1 receptor, type II, membrane receptor
MSR1	Macrophage scavenger receptor 1, membrane receptor
NOS2A	Nitric oxide synthase 2A (inducible), cytoplasmic enzyme
NLGN1	Neuroligin 1, membrane receptor (neuronal)
NPY1R	Neuropeptide Y receptor Y1, membrane receptor (G-protein coupled)
NR4A3	Nuclear receptor (subfamily 4, group A, member 3), steroid-thyroid hormone receptor superfamily
PLAUR	Plasminogen activator, urokinase receptor, membrane receptor
RGS1	Regulator of G-protein signaling 1, membrane regulatory protein
ST3GAL3	ST3 beta-galactoside alpha-2,3-sialyltransferase 3, membrane glycosaltransferase enzyme

TNF	Tumor necrosis factor (TNF superfamily, member 2), cytokine

4. Additional analysis was carried out, using pathway analysis. (Ingenuity Pathways Analysis, Ingenuity Systems, www.ingenuity.com). (Refer to Figures 1-4, below) These analyses are done in terms of the so-called canonical pathways, which are pathways sufficiently well-defined to be considered known and proven. Ingenuity Pathways lists more than 160 pathways. In these experiments, significant responses were seen in around 25 pathways. Figure 1 indicates the seven pathways in which the greatest number of genes were up-regulated or down-regulated in response to shock. These are arranged in order of the pathway responses to shock without resuscitation, at 30 minutes. However, the graphs include all of the groups and time intervals. A number of points should be made.

   a. The response in the sham group was much greater than anticipated. This indicates that the combination of anesthesia and the surgical preparation is itself a powerful stimulant to the organism.

   b. The response to shock, shock plus resuscitation, and shock plus resuscitation plus arginine was different in degree from the response in the sham group, but not in pattern. That is, the pathways showing response to shock also showed response to the surgical preparation itself. This is illustrated in Figure 1.

   c. Within each pathway, there is a distinctive pattern of upregulation and downregulation. Figures 2 through 4 illustrate this, showing a portion of the Hepatic Fibrosis / Hepatic Stellate Cell Activation pathway. In each pathway, the blue color represents down-regulation, and the yellow color represents upregulation. The occasional "mixed" color means that more than one gene contributes to the box on the pathway representing an enzyme, or a receptor complex, but the predominant color represents the predominant response..
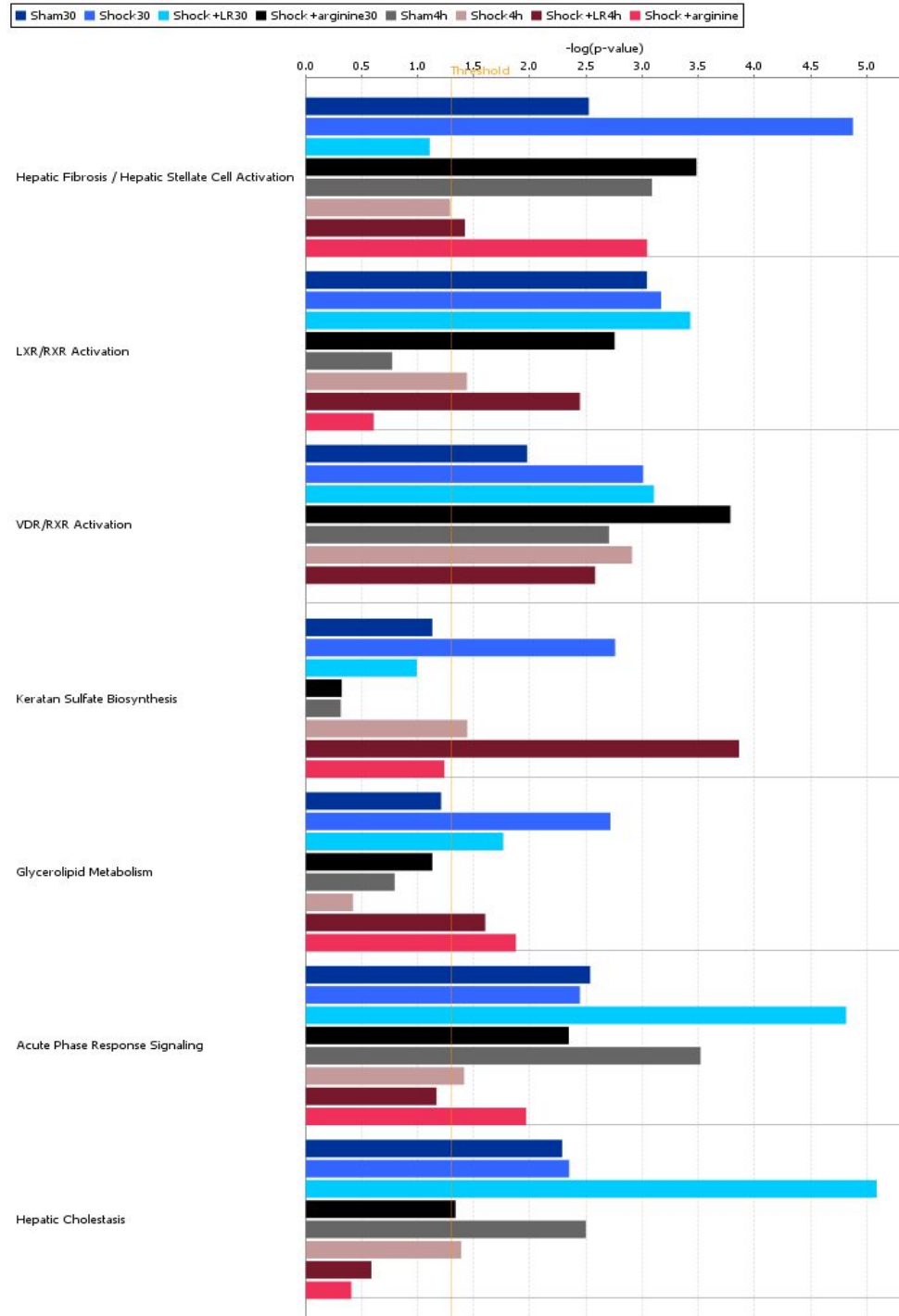
Figure 1: Relative activation of genes in the seven pathways most affected by shock, compared in each group with the baseline values for that group.   The first four bar graphs for each pathway represent sham 30 minutes, shock 30 minutes, shock resuscitated with LR at 30 minutes, and shock resuscitated with LR and arginine at 30 minutes. The second four bar graphs represent the same experimental groups at 4 hours. Most of the pathways show an attenuated response at four hours.

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Figure 2 A portion of the Hepatic Fibrosis/Stellate Cell Activation pathway, showing the hepatic stellate cell, with genes activated in the extracellular fluid, the cell membrane, and the nucleus. Here, the yellow color indicates up-regulation, and the blue color down-regulation. This shows the response to shock without resuscitation at 30 minutes, as compared to baseline for that group.

Figure 3. A portion of the Hepatic Fibrosis/Stellate Cell Activation pathway, showing the hepatic stellate cell, with genes activated in the extracellular fluid, the cell membrane, and the nucleus. Here, the yellow color indicates up-regulation, and the blue color down-regulation. This shows the response to shock with resuscitation using Ringer's lactate at 30 minutes.
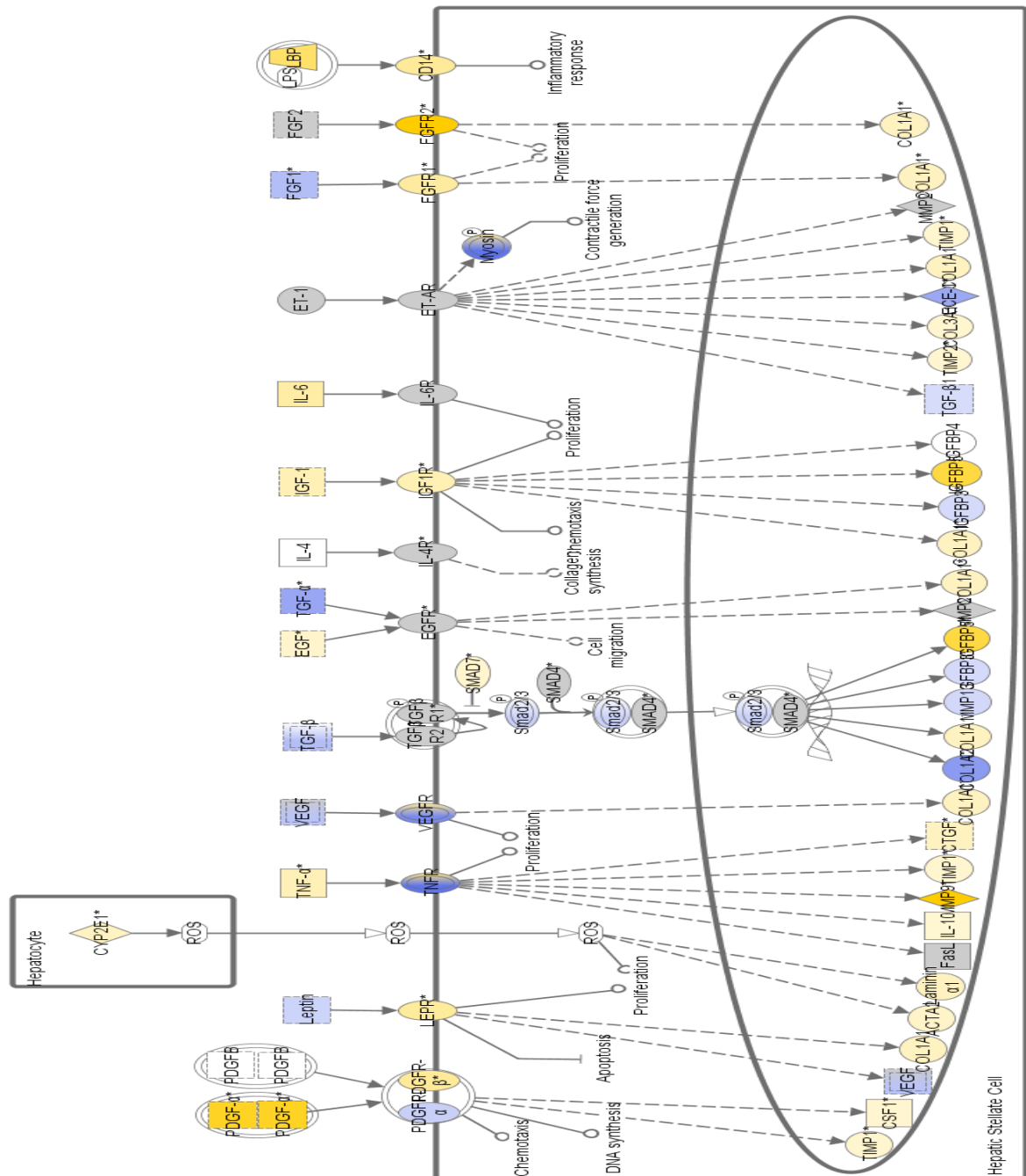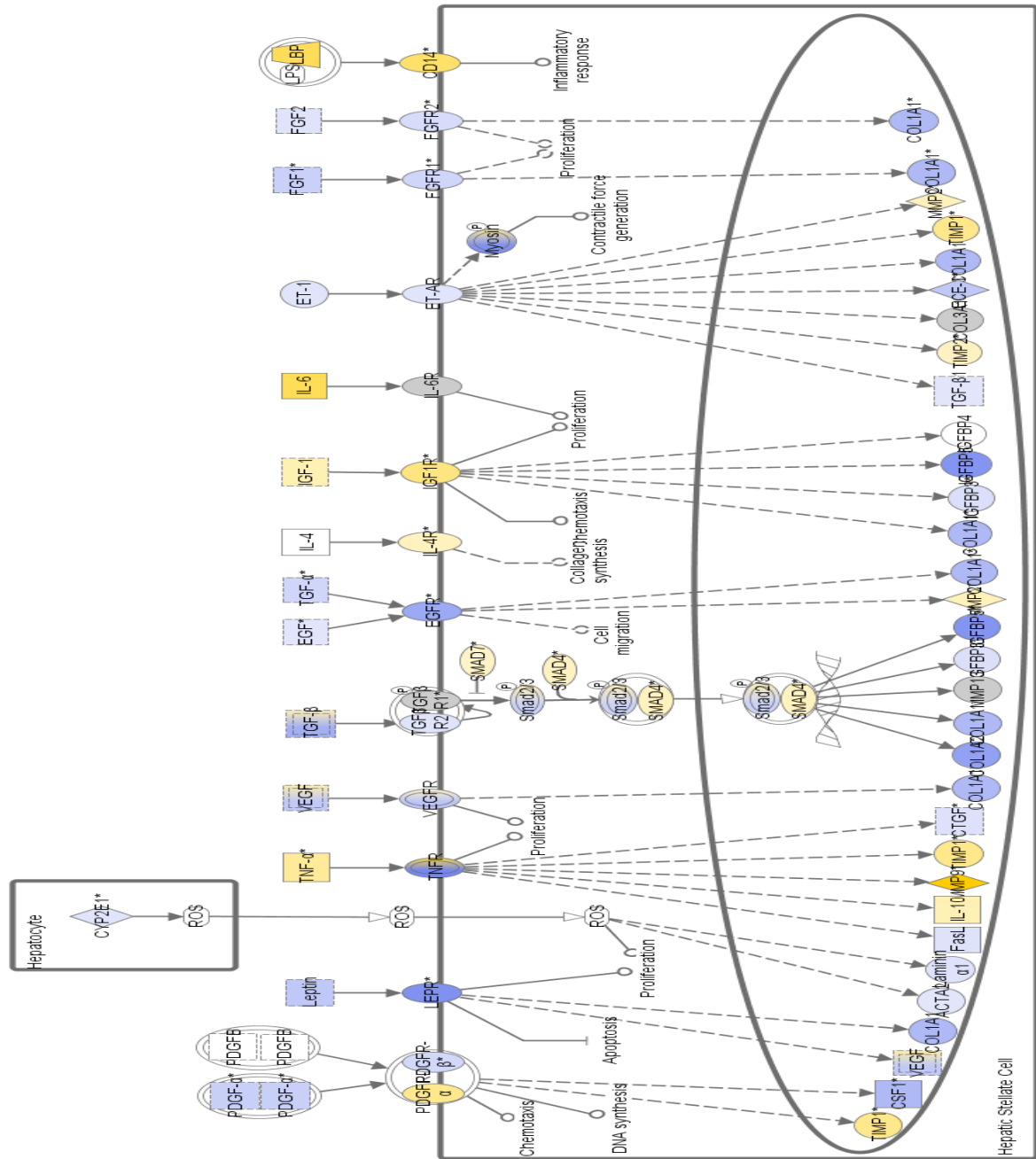
Figure 4. A portion of the Hepatic Fibrosis/Stellate Cell Activation pathway, showing the hepatic stellate cell, with genes activated in the extracellular fluid, the cell membrane, and the nucleus. Here, the yellow color indicates upregulation, and the blue color downregulation. This shows the response to shock with resuscitation and arginine, at 30 minutes, as compared to baseline.

5. Studies using dietary enrichment with omega-3 fatty acids were completed (funded by another grant) and have been reported at a research workshop and at a national meeting. The relevance of these studies is that these compounds are

available in Europe as an intravenous preparation, and they could potentially be used as a component of resuscitation therapy.

6. The figure below (Figure 5) illustrates the experimental model developed for use in subsequent experiments and in the future. It has been modified somewhat from the model used in the above experiments, in which animals were monitored for four hours following the end of the shock period. The particular series of experiments illustrated used the dipeptide alanine-glutamine dipeptide, which is a glutamine equivalent used clinically in Europe and elsewhere (Dipeptivin). Sprague-Dawley male rats, 300-350 grams, were anesthetized with Isoflurane. Cannulation of the femoral artery and vein were carried out. (19) Hemorrhagic shock was induced by withdrawing blood (0.5 ml/min) to lower the blood pressure to 25-30 mm, and maintaining it at that level for 30 minutes. Resuscitation was by lactated Ringer's solution, 21 ml/kg over 30 minutes. Animals were sacrificed 1 hour after the end of the shock period, and blood withdrawn. This model differs in important respects from the model used in the arginine studies described above, in that only one sampling will be done, at 1 hour, rather than at 30 minutes and 4 hours. The original model showed fairly clearly that later sampling showed similar patterns of gene activation as the early sampling, but appeared more attenuated.
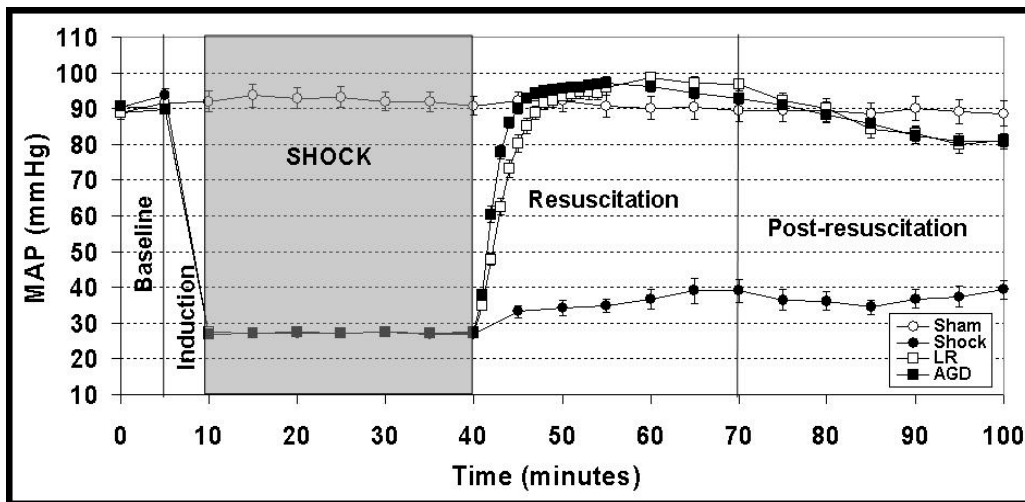

Figure 5. Time course of shock and resuscitation in animal model.

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II. Research objective #2: Define the effect on gene expression of the agents which will be used clinically, using the animal model. *Deliverable*: The effect of the agents upon the level of expression of the signature genes.

1. The agents which have been proposed to be used clinically agents are allopurinol, glutamine, and arginine. Glutamine is an amino acid that has been approved for use in humans. Arginine is also an amino acid which strongly ameliorates the inflammatory reaction, and has shown benefit in the rat model. Allopurinol is available in an intravenous form, and will be used in the further studies. This drug was shown to provide survival benefit in the canine shock model 20 years ago, by the PI and others (1). It appears to act by inhibiting xanthine oxidase, and hence lowering the production of free radicals during reperfusion. Survival studies with each of these agents have been carried out. Current studies were done in the rat model for glutamine (20, 21) and arginine. Previous work by the PI has shown survival benefit for allopurinol in both the canine shock model and the rat model. Previous studies were also done on alanine-glutamine dipeptide (21), crocetin (22), and dehydroepiandrosterone (7).

Glutamine studies are presented in this table.

Survival with or without Glutamine in Resuscitation

	LR	Glutamine
Rat weight (gram)	295.83±3.03	293.00±3.84
Cannulating Time (minutes)	6.50±0.29	6.50±0.23
Bleeding (ml)	9.22±0.41	9.05±0.58
***Survival > 24 hours**	4/12	11/12

Shock at MAP = 22±4 mmHg, 90 minutes. Resuscitated with Ringer's Lactate (LR, 21ml/kg) with/without glutamine (630mg/kg) over 30 minutes. Data expressed as mean ± SEM. *P< 0.05 (LR vs Glutamine).

Arginine studies are presented here. In this model, arginine was nearly as effective as glutamine.

Survival with or without Arginine in Resuscitation

	LR	Arginine
Rat weight (gram)	286.70±4.03	294.40±4.81
Cannulating Time (minutes)	6.35±0.13	6.80±0.17
Bleeding (ml)	9.32±0.31	10.06±0.35
***Survival < 24 hours**	11/20	4/20
Survival > 72 hours	6/20	12/20

Shock at MAP = 20±4 mmHg, 90 minutes. Resuscitated with Ringer's Lactate (LR, 21ml/kg) with/without arginine (300mg/kg) over 30 minutes. Data expressed as mean ± SEM. *P< 0.05 (LR vs Arginine).

    a. The genome data from the shock experiment described in Objective #1 was analyzed further, specifically in the analysis of the two shock groups treated with and without arginine. The objective was to determine the

differential effect of adding arginine. Also shown here is the relative activation of pathways as measured by up-regulation of genes in those pathways. This data is presented above, in Figures 1-4, and explained in more detail under paragraph I.4. It is very clear that the treatment affects the response of the genome. There is considerable difference in the gene responses within the pathway, depending on the experimental condition. Figure 2 can be seen the response to shock alone. Figure 3 shows the response to shock with resuscitation using Ringer's lactate. And Figure 4 shows the response to shock with resuscitation with added arginine. In Figure 1, it can be seen that the detailed response of genes in this pathway is different for the three conditions (bars #2, 3, and 4, respectively). The patterns of gene up-regulation and down-regulation change with the treatment.

2. A cell-culture based model has been developed, using a standard macrophage cell line (RAW 264.7). This model is based on exposing cultured macrophages to ischemia in a sealed chamber, and then re-exposing them to levels of oxygen found in room air (21%) The intent of this model has been to reproduce the hypoxic insult produced in shock by reduced tissue perfusion. Macrophages were selected because they are one of the major cell types that regulate the inflammatory response. Since tissue oxygen partial pressure is normally quite low compared with atmospheric, in the range of 20 mm Hg, reduction of the oxygen level to below that was necessary to produce a response. We found that reducing the level of oxygen to 1.5% from the usual 21% produces a significant hypoxic insult. This corresponds to a partial pressure of oxygen of 10 mm Hg. Measuring the effect of therapeutic agents on hypoxia-challenged cell lines, will provide further evaluation of their possible therapeutic effectiveness. Several significant preliminary observations have been made.

   a. Cells grown in hypoxia grow at a rate about 15% less than cells in normoxia for 24 hours, and their growth slows markedly thereafter as compared with normoxic controls.

   b. Adenosine triphosphate (ATP) levels within the cells were measured using a luciferase assay. Data indicates that ATP in cells left chronically ischemic for up to 24 hours have the same ATP content per cell as cells grown in normoxia. This observation is being subjected to further study with apparatus that allows actual measurement of oxygen partial pressure in the cell culture medium. The relationship between hypoxia and ATP production appears to be time-dependent, with cells initially showing a drop in ATP, then a subsequent recovery over 4-8 hours, and finally a drop after 10-12 hours.

   c. Hypoxia produces a differential response to iNOS. Ischemic cells show an increase in mRNA for iNOS (by RT-PCR), but no increase in nitric oxide activity, and no increase in protein content (by Western blot).

   d. Studies strongly suggest that stress-induced modifications of host responses are associated with a change in the proteolytic subunits of the multifunctional cellular proteasome. This was confirmed in the hypoxic macrophage model, in which the chymotrypsin-like activity, but not the trypsin-like or post-glutamate activities, was increased in hypoxia. This change in activity was

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found to be responsive to changes in arginine concentrations, but independent of glutamine concentrations.

e. In analyzing the protein subunits that comprise the proteasome, we found over-expression of LMP2 protease as compared with constitutively-expressed X protease. Moreover, this over-expression was responsive to changes in arginine concentrations. In the Western blot below, bands reflect relative levels of LMP2 protease and the numbers indicate the arginine concentration in culture media. Level of LMP2 protein appears to be highly responsive to the concentration of arginine.
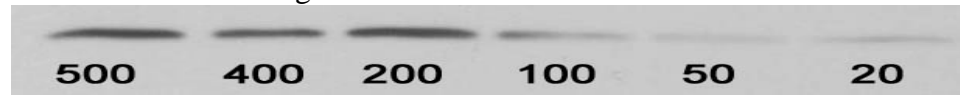
This appears to be dependent upon which agent is employed, as LMP2 levels are not responsive to varying the concentration of glutamine.

f. The proteasome is central to many of the cell's immune functions, and to its response to inflammatory stimuli, including MHC-directed antigen presentation and regulation of cytokine production. Arginine concentration appears to be important in regulation of proteasomal activity. This underscores the concept that we expect to develop to maturity in further research, namely that changes in levels of arginine and/or glutamine at the level of the cell can profoundly affect the inflammatory response at the cellular level through changes in the proteasome.

g. Grant funding to carry this line of research further is being sought. In addition, expansion is planned to the study of intestinal epithelial cells (Caco2) and to hepatic cells (HEP-4).

Research objective #3: Assess gene expression in patients treated with current initial resuscitation protocols. Deliverable: Coordinated with objective #1, A panel of genes that are reproducibly altered in white blood cells and in liver and muscle by shock and resuscitation.

1. The experimental protocol was approved by the Institutional Review Board of the University of Missouri – Kansas City. It was reviewed and approved by the Human Research Protection Office (HRPO), Office of Research Protections (ORP), U.S. Army Medical Research and Materiel Command (USAMRMC).

2. Under this protocol, blood samples were taken from patients arriving in the trauma center who are injured severely enough to exhibit blood loss or signs of hypovolemia. Blood samples were collected within one hour following resuscitation, again at 24 hours, and at 7 days or time of discharge from hospital, whichever comes first. These samples are then processed to isolate the white blood cells and extract the RNA, and then submitted for microarray analysis. A total of 15 patients have completed the study.

3. Several conclusions can be drawn. First, the genomic response is maximal at the first hour following resuscitation, with progressively less response at 24 hours and at 7 days or discharge day. Second, there is broad response across the genome, but large responses (four-fold up or down) are confined to a relatively smaller group of genes.

Third, many of the genes involved in inflammation are highly up-regulated following trauma.

4. There have been a number of limitations to this study. First, the requirement for obtaining informed consent for the study has limited enrollment to patients with only mild to moderate trauma and blood loss. A further limitation is that only those patients whose mental faculties are intact can participate in the consent process. It has not been possible to enroll patients with severe shock, because there is no time for an adequate informed consent before the patient must be taken to the operating room. Typical time from emergency department admission to operating room is around 10 minutes in severely injured patients. Second, sample processing has been difficult, because the method initially used for white blood cell isolation was time-consuming and technically difficult. Since many patients arrive at night or on weekends, it has been difficult to carry out the processing. However, a commercially available white blood cell isolation system has been introduced. (LeukoLOCK Total RNA Isolation System, Ambion, Austin TX). This system allows the investigators to extract the white blood cells using a simple filter technique, inactivate the cells using a commercial hypertonic preservation agent (RNAlater, Ambion, Austin, TX), and keep the sample refrigerated for several days without degradation or alteration of the mRNA. This has greatly simplified initial processing, and has resulted in good enrollment to the study.

5. Data analysis has been in terms of the initial and 24-hour samples being compared with the 7-day (or discharge) samples. This has been a very useful way to analyze the data, since each patient is, basically, his or her own control. Alternatively, we have analyzed the data by using pooled 7-day samples as controls for the entire group. This has allowed inclusion of several patients who failed to return for the 7-day study. In future research in this area, we propose to obtain samples on a group of 10 normal volunteers, to act as a population control. This group will be selected from hospital and university personnel, from volunteers, and will be selected to match the age and sex distribution of the trauma patients – i.e., age 20 to 40, 80% men. But this refinement is beyond the scope of the present study.

6. Analysis of the data shows several patterns very similar to the results seen in the rat model. The following table documents twenty genes, all of which appear to be elevated following injury. Of note, several of these are the human equivalents to the genes found to characterize the response to shock and injury in the rodent model.

Gene	Protein and function
ALOX5	Arachidonate 5-lipoxygenase (cytoplasmic enzyme)
B3GALT3	Membrane-bound galactotransferase enzyme
B3GNT1	Galactotransferase enzyme
CA4	Carbonic anhydrase 4; cell surface bound enzyme
CLEC4D	C-type lectin domain family 4; regulatory protein
CXCL5	Chemokine (C-X-C motif) ligand 5, chemokine, cytokine
F5	Coagulation factor, membrane receptor
GPR30	G-coupled membrane estrogen receptor
HSPA1B	Heat shock (70kDa) protein 1b
HSPA6	Heat shock (27kDa) associated protein 6

IL1RN	Interleukin 1 receptor antagonist
IMP3	Small neucleolar riboprotein
JAK2	Janus kinase 2; JAK-STAT pathway
PLAUR	Plasminogen activator; urokinase membrane receptor
PTGFR	Prostaglandin F receptor
SLC1A3	Glutamate aspartate membrane transporter
SOCS3	STAT-induced STAT inhibitor; cytokine regulatory protein
ST3GAL6	Membrane glycosaltransferase enzyme
THEDC1	S-acyl fatty acid synthase thioesterase
TNFSF13B	Tumor necrosis factor ligand (TNF superfamily); cytokine
TREML4	Immunoglobulin trans-membrane receptor

Research objective #4: Assess the response of patients to administration of modified resuscitation procedures. Deliverable: The effect of the experimental agents upon signature genes, cellular response, and cellular damage in patients.

1. All three potential agents, arginine, glutamine, and allopurinol, have shown improvement in survival in whole-animal models of shock, all can be given intravenously, and all are safe when given to humans in the doses contemplated. However, responding to guidance at the Trauma Portfolio Review (Product Line Review, San Antonio, TX, January 6, 2010), we modified the study to employ only one of the three agents, specifically glutamine. It was felt by the review panel that trying to study all three agents initially would result in data which would be difficult to interpret, and would not be approved by the Human Research Protection Office (HRPO). Nor, it was noted, would such a study be useful for possible subsequent FDA submission. After carefully reviewing our previous work and the relevant literature, we selected glutamine for human use. It should be noted that none of these three agents have been used clinically in post-injury treatment before. However, glutamine has been used more extensively than either of the other two agents in critically ill patients. Optimal dosage has already been established by other investigators, and it would seem reasonable to use the most-used agent for this novel application.
2. Our intent has been to evaluate the response of patients to glutamine as a component of resuscitation, using a prospective, randomized, double-blind model, and to study the response in terms of inflammatory genes.
3. The study has been approved by the Institutional Review Board of the University of Missouri - Kansas City, and has been approved by the Human Research Protection Office (HRPO), Office of Research Protections (ORP), U.S. Army Medical Research and Materiel Command (USAMRMC). It has been re-submitted to the UMKC IRB for final approval prior to entering patients. This approval was granted, and the study has been begun.
4. Initial clinical studies show that the glutamine is well-tolerated. Studies of gene regulation have not been completed, and will be completed under subsequent contract funding.

REPORTABLE OUTCOMES

PUBLICATIONS AND PRESENTATIONS

1. Yang R, Tan X, Thomas AM, Shen J, Qureshi N, Morrison DC, Van Way CW 3rd. Crocetin inhibits mRNA expression for tumor necrosis factor-alpha, interleukin-1-beta, and inducible nitric oxide synthase in hemorrhagic shock. JPEN J Parenter Enteral Nutr. 2006 Jul-Aug;30(4):297-301.

2. Yang R, Tibbs BM, Chang B, Nguyen C, Woodall C, Steppacher R, Helling T, Van Way CW 3rd. Effect of DHEA on the hemodynamic response to resuscitation in a porcine model of hemorrhagic shock. J Trauma. 2006 Dec;61(6):1343-9.

3. Yang R, Van Way CW III, Tan X, Thomas A, Shen J, Steppacher R, Qureshi N, Morrison DC. Alanine-glutamine dipeptide inhibits expression of inflammation-related genes in hemorrhagic shock. Clinical Nutrition Week of the American Society for Parenteral and Enteral Nutrition, Dallas. February 12-15, 2006. (Oral presentation). Clinical Nutrition week,. 2006, p145. (Abstract of Distinction)

4. Thomas A, Yang R, Van Way CW III, Quresh N. A Preliminary micro-array study of the response to hemorrhagic shock and to therapy with crocetin and alanine-glutamine dipeptide. Kansas City Area Life Sciences. March 30, 2006. Kansas City Life Sciences. 2006, p163. (Abstract)

5. Yang R, Van Way CW III, Tan X, Thomas AM, Shen J, Steppacher R, Qureshi N, Morrison DC. Alanine-glutamine dipeptide inhibits inflammatory mediator genes in hemorrhagic shock. Kansas City Area Life Sciences. March 30, 2006. Kansas City Life Sciences. 2006, p153. (Abstract)

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CONCLUSION

This study has rested on two basic hypotheses. The first is that agents given during and (for a short period of time) after resuscitation can favorably influence the response to hemorrhagic shock and injury. The second is that studies of gene response studies, specifically microarray and RT-PCR studies, could be used to assess the response of patients being treated for injuries to such therapeutic agents.

To date, both hypotheses have been shown to be true in the animal model. Work continues to determine optimum therapeutic agents to be used during and after resuscitation. Clinical studies using glutamine have been successfully carried out. They are being continued using other support.

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1 **Appendix A: Summary of Gene Activation Studies**

2 **Gene Activation in White Blood Cells Following Arginine Administration**

3 **for Experimental Hemorrhagic Shock**

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15

16

17

1 **Abstract**

2 **Introduction**: Administration of L-arginine has been shown to significantly modify the

3 host inflammatory response to stress in rats. This study was carried out in an experi-

4 mental animal model of hemorrhagic shock to determine the therapeutic benefit of L-

5 arginine as an addition to a standard resuscitation regimen in increasing survival by

6 modulation of inflammatory gene activation.

7 **Methods**: To assess survival after hemorrhagic shock, 40 Sprague-Dawley male rats

8 (300 gm) were subjected to 90 minutes of shock at 25 mm Hg, then resuscitated over

9 30 minutes with 6 ml/kg Ringer's lactate (LR) either with or without added L-arginine

10 (300 mg/kg). To evaluate host inflammatory gene responses, 16 rats were divided into

11 4 groups (sham, shock alone and shock resuscitated with Ringer's lactate with or with-

12 out arginine). A 30 minute shock period was used (This is inconsistent with the previ-

13 ous statement which indicates 90 minutes!). In the resuscitation groups, 28 ml/kg Ring-

14 er's lactate was administered over 30 minutes, with or without L-arginine at a concentra-

15 tion of 300 mg/kg.   At baseline and 30 and 240 minutes post resuscitation, blood was

16 drawn, white cells isolated, RNA extracted, and microarray analysis carried out.

17 **Results:** Survival was significantly improved in the L-arginine-treated group of rats

18 relative to control resuscitation treated animals. Survival (24 hours) in the rats in the

19 control and L-arginine groups was 9/20 and 16/20 (p<0.05) respectively. In the microar-

20 ray studies, significant gene activation within 26 separate canonical pathways was de-

21 tected in shock rats resuscitated with Ringer's lactate. Inclusion of L-arginine in the re-

22 suscitation fluid was reproducibly associated with increased levels of gene activation in

23 5 additional pathways and decreased levels of activation in 6 pathways: A group of 20

1 'signature' genes was identified that may reflect improved host responsiveness to ther-

2 apeutic intervention and survival. .

3 **Conclusions:** Administration of L-arginine with resuscitation fluids improved survival

4 from lethal hemorrhagic shock.   Results of analysis of gene activation indicate that ar-

5 ginine differentially affects 11 metabolic and signaling pathways, as compared with

6 controls suggesting a potential correlative relationship with survival.

1 **Introduction**

2   L-Arginine appears to play a critical role in the development/regulation of the host

3 inflammatory response to stress or trauma. It is a nitrogen donor in a number of bio-

4 synthetic reactions. It also serves as the substrate for inducible nitric oxide synthase,

5 which is intimately involved in the development of an inflammatory response and is up-

6 regulated in response to a number of stressful stimuli. L-Arginine deficiency impairs

7 nitric oxide (NO) synthesis, both in normal animals and in animals challenged with lipo-

8 polysaccharide (LPS).[1] In this respect, there is strong evidence that the decreased

9 availability of L-arginine correlates with reduced levels of expression of the inducible ni-

10 tric oxide synthase (iNOS) enzyme, probably through reduced levels of translation of

11 mRNA for iNOS.[2] L-Arginine appears to be involved directly in regulation of iNOS ac-

12 tivity, and is more than just as a substrate for the production of NO.[3]

13   Previous research on the potential therapeutic use of L-arginine as an adjunct to

14 resuscitation in experimental hemorrhagic shock is somewhat sparse,[4-10] but results of

15 the available literature collectively allow the conclusion that L-arginine supplementation

16 can be beneficial in ameliorating the tissue damage produced following experimental

17 hemorrhagic shock. In the present investigation, we have critically evaluated the contri-

18 bution of L-arginine as a potentially beneficial component of the resuscitation fluid in an

19 experimental rat model of hemorrhagic shock. Our studies were designed to test two

20 hypotheses. First, L-arginine supplementation during resuscitation will improve survival

21 from lethal hemorrhagic shock. Second, the overall patterns of gene expression ob-

22 served in white blood cells following shock will be reflective of changes in metabolic and

23 signaling pathways primarily affected by shock and resuscitation that are significantly

1   influenced by treatment with L-arginine. We postulate that the latter may potentially al-

2   low identification of host genes that may significantly influence survival from lethal

3   shock.

4

1 **Materials and Methods**

2 **Experimental Hemorrhagic Shock**

3 *Gene Expression Studies*: Male Sprague-Dawley rats, 280-300 gm were pur-

4 chased from Charles River, Inc (Boston, MA.) Animals were anesthetized with isoflu-

5 rane (Baxter Healthcare, Deerfield, IL), using a standard vaporizer (SurgiVet, Inc.

6 Waukesha, WI).   Oxygen was administered at 3L/min.   Using aseptic technique, the

7 femoral artery and vein were cannulated using Polyethylene catheters (PE50; Clay Ad-

8 ams, Becton Dickinson and Company, Sparks, MD). Blood pressure and heart rate

9 were monitored continuously by connecting the femoral artery catheter to a pressure

10 transducer and computerized system (Digi-Med, Micro Med Inc, Louisville, Kentucky),

11 with continuous monitoring.   An initial blood sample was taken for analysis, and then

12 shock was produced by withdrawing blood from the femoral artery until the mean arteri-

13 al pressure (MAP) was 25 mm Hg. Blood pressure was maintained at 25-30 mm Hg for

14 30 minutes. Depending on the experimental group (see below), resuscitation was car-

15 ried out by infusing 21 ml/kg Ringer's lactate (LR) over 30 minutes, either with or without

16 supplemental L-arginine, 300 mg/kg. Racemic LR solution was used (Hospira, Inc,

17 Lake Forest, IL). L-arginine was added to the LR in powdered form (Acros Organics,

18 New Jersey), using filtration to sterilize. Following this, half of the animals were kept

19 anesthetized for 30 minutes, and then sacrificed by aortic transection after obtaining a

20 blood sample.   The other half of the animals had the catheters removed and incisions

21 closed, and then were allowed to waken. At four hours post shock, they were re-

22 anesthetized using Isoflurane, a blood sample was drawn, and they were sacrificed by

23 aortic transection.

1   *Experimental protocol, gene studies:* The rats were randomized into four groups;

2   Sham Group, Shock Group, Shock and lactated Ringers(LR) Resuscitation Group, and

3   Shock and lactated Ringer's with L-ArginineResuscitation Group as described above.

4   The table below indicates the numbers of animals successfully analyzed in each group.

Experimental Group	Baseline	30 minutes	4 hours
Sham	4	3	3
Shock	4	3	3
Shock and LR	5	3	3
Shock, LR and Arginine	3	4	4

5

6   Lethality Studies: In order to determine if L-arginine used during resuscitation affected

7   overall survival, a separate group of animals was studied. Male Sprague-Dawley rats,

8   280-300 gm were used (n= 20 per group). Anesthesia and initial surgical preparation

9   were as described above. The animals were subjected to 90 minutes of shock with a

10   mean blood pressure of 20 mmHg. Resuscitation was as described above, by infusing

11   21 ml/kg Ringer's lactate (LR) over 30 minutes, either with or without supplemental L-

12   arginine, 300 mg/kg.   At the conclusion of the resuscitation phase, the catheters were

13   removed, the incisions closed, and the animals were allowed to awaken from anesthe-

14   sia. They were then observed for the next 72 hours, with the end point of the experi-

15   ment being non-survival. After that, the surviving animals were sacrificed.

16   **RNA Preparation and Probe synthesis.**

17

18   Whole blood specimens were collected from individual rats following the proto-

19   cols summarized above in EDTA. Contaminating red blood cells (which lack significant

20   amounts of RNA) were lysed to facilitate their separation from the white blood cells and

21   remove contaminants, including heme and protein. RNA extraction was carried out us-

22   ing the Versagene RNA purification kit (Gentra Systems, Minnesota, USA) using the

1 Protocol for 'RNA Purification' from 3.0-10.0 ml Whole Blood or Buffy Coat Containing

2 12-70 million White Blood Cells. The resulting RNA was confirmed to be essentially free

3 of genomic DNA, protein and other (Other than what?) enzymatic inhibitors.

4   Total RNA was purified with Versagene RNA purification Kit by first adding the

5 white blood cells to a detergent/salt solution to lyse and homogenize the cells and elimi-

6 nate potential endogenous RNase activity. Homogenization disrupts the cell mem-

7 branes releasing RNA into the lysis solution, and shears the genomic DNA decreasing

8 the viscosity of the lysate. Whole blood lysates from blood volumes greater than 3.0 ml

9 were then passed through the Gentra PRECLEAR column to remove the remaining

10 debris after homogenization. Next, the lysates were applied to the purification column to

11 bind the RNA and wash away proteins, DNA and other contaminants. Residual DNA

12 was removed by an on column DNase treatment and then the DNA fragments and

13 DNAase were removed by subsequent washing steps. Finally, the purified RNA was

14 eluted with DEPC-treated water. The extracted RNA was stored at -80 degrees until

15 used for experiments.

16

17 **Array Hybridization**

18   Samples for analysis of gene expression levels were processed using the Affy-

19 metrix standard Target Labeling Protocol,and were carried out at the Microarray facility

20 at University of Kansas Medical Centre (KUMC). Prior to experiments, all RNA samples

21 were analyzed for RNA quality and quantity using the Agilent 2100 Bioanalyzer.

22   The RNA was initially annealed using T7(dT)24 primer, which was then reverse

23 transcribed into cDNA using the Superscript Choice system (Invitrogen).After the first

1   and second strand synthesis, the cDNA extraction was carried out followed by Phase

2   Lock Gel separation (Brinkman Instruments/Eppendorf) and then by DNA precipitation.

3   Invitro-Transcription Biotin Labeling was achieved using the GeneChip Expression 3'-

4   Amplification IVT Labeling kit. The cRNA generated from either IVT labeling reaction

5   was purified using RNeasy RNA Purification Mini kit (Qiagen) using the RNA Cleanup

6   Protocol according to the manufacturer's instructions. The Biotin- labeled cRNA was

7   quantitated using 260nm/280nm spectrophotometric assays.

8   The GeneChip was pre-hybridized with 200ul 1x Hybridization buffer at

9   45C for 10min at 60rpm.   Then 200ul denatured Hybridization cocktail was applied to

10   the GeneChip, which was then hybridized for 16 hr at 45C and 60rpm in the GeneChip

11   Hybridization Oven 640. Hybridized GeneChips undergo low and high stringency wash-

12   ing and R-Phycoerythrin-Streptavidin staining procedures using the GeneChip fluidics

13   station 450.. Scanning was conducted using the Agilent Gene Array Scanner. 1x scans

14   were conducted for the GeneChip Expression 3'-Amplification IVT Labeling using a Pix-

15   el value = 3um and wave length of 570nm. Absolute and comparison analysis were

16   conducted using the following settings

17   Scaling - All Probe Sets: Target Signal = 500

18   Normalization – User Defined: Scale Factor = 1

19   Data Quality Control Analysis was done using Affymetrix GeneChip operating software

20   (GCOS).

21   **Data Analysis**

1   The estimates of the potential networks involved and functional analyses were

2   evaluated using the Ingenuity Pathways Analysis (Ingenuity Systems,

3   www.ingenuity.com).

4   **Network Analysis Generation**

5   *1. Generation of the Data Set.*

6   The data set containing gene identifiers and corresponding Expression Values

7   were uploaded into the Ingenuity Pathways software application. An Expression Value

8   is defined as a numerical value that indicates the extent of activity (or relative im-

9   portance) of each component (specific gene expressed) of the pathway being queried.

10   We used 'ratio' as the expression value type. Each gene identifier was mapped to its

11   corresponding gene object in the ingenuity Pathways Knowledge Base. A ratio thresh-

12   old of 3.0 was established to distinguish genes whose level of expression was signifi-

13   cantly differentially regulated relative to controls. These genes, called focus genes, were

14   overlaid onto a global molecular network developed from information contained in the

15   Ingenuity Pathways Knowledge Base. Networks of these focus genes were then algo-

16   rithmically-generated, based on the extent of their connectivity.

17   *2. Functional Analysis of the Entire Data set.*

18   The Functional Analysis approach was used to identify the biological functions

19   and /or associated disease states that were most significant with respect to the ob-

20   served data set. .Only those individual genes from the data set that satisfied the

21   threshold cut off of 3, and were associated with biological functions and/or disease state

22   of the Ingenuity Pathways Knowledge Base were subsequently considered for analysis.

23   Fischer's exact test was used to calculate ap – value determining the probability that

1 each biological function and /or disease state assigned to that data set was due to

2 chance alone

3 *3. Canonical Pathway Analysis:*

4   Canonical pathways are well-characterized metabolic and cell signaling path-

5 ways. Using the Ingenuity Pathways analysis library, the canonical pathways that

6 most significantly correlated with the generated data set. Genes from the data set that

7 satisfied the threshold ratio cut off of 3 and were associated with a canonical pathway

8 in the Ingenuity Pathways Knowledge Base were considered for analysis. The signifi-

9 cance of the extent of the association between the actual data set and the specific ca-

10 nonical pathway was evaluated in 2 ways. First, the ratio of the number of genes identi-

11 fied to be significantly regulated and that map to a specific canonical pathway was dis-

12 played. Second, Fischer's exact test was used to calculate a p- value determining the

13 probability that the observed association between the identified genes in the data set

14 and a specific identified canonical pathway could be explained by chance alone.

15

16 **Results**

17 **1. Survival following hemorrhagic shock by treatment with L-arginine**

18   We first assessed the extent to which administration of L-arginine in the resusci-

19 tation fluid (Ringer's Lactate) would promote overall survival up to 72 hours post-shock.

20 Survival was observed to be 80% in the group of rats administered L-arginine as com-

21 pared with 45% in the control group of rats (p<0.05). Survival at 72 hours was 60% ver-

22 sus 30% (p=0.06) respectively. ifferences between the groups of rats in weight, cannu-

23 lation time, and amount of blood loss were small and non-significant (Table I). The rats

1 in the L-arginine group showed a small, but non-significant, increase in the return of

2 blood pressure at the end of resuscitation (Figure 1). We conclude the inclusion of L-

3 arginine in the resuscitation fluid has a statistically significant early beneficial and pro-

4 tective effect against mortality following hemorrhagic shock

5 **2. Gene Activation Following Shock and Resuscitation- effect of L-arginine**

6 To correlate changes in survival with events occurring at the molecular level, we moni-

7 tored the rat genome for changes in level of expression of genes activated in response

8 to hemorrhagic shock and reperfusion. The microarray analysis used in this study al-

9 lowed us to monitor 31,099 genes that were either up- or down-regulated in peripheral

10 blood mononuclear cells following shock and resuscitation As summarized by   the data

11 in Table II, it is clear that even the sham surgical procedure provided a strong stimulus

12 in modulation of the gene response in peripheral blood mononuclear cells.   Neverthe-

13 less, the overall pattern of the rat host response to hemorrhagic shock that remained

14 untreated, and to hemorrhagic shock followed by either type of resuscitation was quali-

15 tatively similar at least as assessed quantitatively by the total number of genes affected.

16 **3. Pathway analysis of relative Gene Expression**

17   In order to provide more insight into exactly which genes were affected by the

18 hemorrhagic shock treatment and subsequent intervention, detailed analysis of the ca-

19 nonical pathways was undertaken, This type of analysis allows information on the vari-

20 ous metabolic and signaling pathways that are affected by the shock to be determined

21 as well as on the consequences of treatment with lactated Ringers supplemented with-

22 arginine relative to lactated Ringer's alone. The most affected pathways at both thet 30

23 minute and 4 hour post shock treatment periods are summarized in Table III and sup-

1 port the conclusion that L-arginine affects multiple pathways leading to the observed

2 protective effects.

3   Analysis of the specific effects of L-arginine on individual genes carried out using

4 network analysis profiles.   These were constructed for each of the several major path-

5 ways, using different color-coding for up-regulation and down-regulation. From these

6 profiles, analyses of the activation signaling pathways for a number of the major cyto-

7 kines were carried out and a detailed listing is presented in Table IV. Table IV includes

8 only those genes that had a fold change of more than threefold and a differential of

9 more than threefold from that of the shock group. These analyses, based as they are on

10 the analysis of only a relatively small number of animals, need to be regarded as quali-

11 tative profile rather than a quantitative representation.   Nevertheless, as expected, giv-

12 en the prominent role of inflammation in mediating much of the post resuscitation dam-

13 age to tissues observed following shock, many of the gene expression profiles influ-

14 enced by L-arginine are associated with inflammatory cytokines and/or acute phase re-

15 sponses.

16 **Discussion**

17   The results of the studies outlined in this manuscript are fully consistent with the

18 perspective that a critical time following hemorrhagic shock is the period during which

19 the patient is being resuscitated, a time at which   a massive stimulation of the inflam-

20 matory response occurs which, in turn, produces the cellular and organ damage that is

21 so deleterious to organ function.[8,9] The administration of L-arginine provides protection

22 against these deleterious effects. As suggested by the results of the detailed microarray

23 studies, the manifestations of these protective effects are reflected by changes in the

1   extent of activation of proinflammatory genes. The mechanisms by which these protec-

2   tive effects are mediated at the level of gene expression remain to be determined.

3   These   data support the view, held by many in the field, that so-called "pure"

4   hemorrhagic shock preparations do not exist.   Such preparations are, in fact, invariably

5   a combination of trauma and shock.   The present shock preparation could be consid-

6   ered as relatively minor from a surgical standpoint, but it still required general anesthe-

7   sia, a skin incision, muscle dissection, and insertion of indwelling catheters.   It can be

8   seen from the data in Table II that even the "sham" animals were associated with

9   changes in the expression of a very large number of genes.   Whether this effect could

10   be minimized by pre-placement of the catheters 48 or 72 hours in advance is undeter-

11   mined.   The presence of chronic indwelling catheters and the restraint necessary to car-

12   ry out the shock procedure would, in any case, be likely to provide stressful stimuli.   As

13   the results from this study demonstrate, gene expression is highly sensitive to adverse

14   stimuli, and these definitely include general anesthesia and surgical manipulation.

15   Arginine, as adjunct to resuscitation in the treatment of hemorrhagic shock, has

16   been evaluated by other investigators.   Arora, et al, administered L-arginine in a swine

17   model of shock.[4]   The dose used, 300 mg'/kg, was the same as used in the present

18   study.   The L-arginine-treated animals showed improved arterial pressure, lowered lev-

19   els of lactate, and increased survival relative to controls.   Results of a more recent study

20   by Beenyo et al, in a cat model of hemorrhagic shock, demonstrated   that L-arginine

21   administration reversed the hepatic vasoconstriction induced by shock.[5]   Naloxone had

22   a similar effect.   They suggested that increased nitric oxide synthesis in response to L-

23   arginine might be the mechanism.

1   Extensive studies of L-arginine resuscitation in shock were carried out by An-

2   gele, Chaudry, and co-workers. [6-10]   They used the rat model, bleeding to 40 mm Hg in

3   a modified Wiggers preparation, and resuscitation with 300 mg/kg L-arginine (or saline

4   control) in addition to Ringer's lactate at 4 times the volume of shed blood.[6]   Resuscita-

5   tion with L-arginine increased cardiac output and regional perfusion.[6]   L-Arginine ad-

6   ministration reversed shock-induced depression of splenic blood flow, IL-2 and IL-3 re-

7   lease from splenocytes, and splenocyte proliferation.[7]   L-Arginine restored the shock-

8   induced depression of IL-1β and IL-6 from splenic and peritoneal macrophages, as well

9   as lowering the increased plasma levels of IL-6.[8]   Using a model of wound healing in

10   the mouse, collecting wound immune cells and fluid in polyvinyl sponges, they found

11   that wound immune cells also responded in a somewhat similar fashion.   L-Arginine re-

12   stored the capacity of wound immune wound immune cells to release IL-1β and IL-6.

13   IL-10 was increased in the wound fluid in animals receiving L-arginine.[9]   In a study of

14   liver injury, L-arginine attenuated the shock-induced increase in plasma glutathione S-

15   transferase (α-GST) and prevented neutrophil infiltration and liver edema.[10]

16   There is some evidence that resuscitation with L-arginine cam improve wound

17   healing following hemorrhagic shock. A study by Wittmann, Chaudry, Angele, et al, in

18   C3H/HeN mice, showed that resuscitation with L-arginine restored the shock-induced

19   depression of hydroxyproline, a collagen metabolite, and prevented depression of colla-

20   gen I synthesis.   The L-arginine-treated mice showed increased wound breaking

21   strength as compared with controls. They concluded that L-arginine resuscitation im-

22   proved wound healing by increasing collagen synthesis.[11]   Shi, et al, used a rat model,

23   bleeding to 30 mm Hg for 90 minutes, resuscitation with Ringer's lactate at 3 times the

1   volume of shed blood.[12] Wounds were placed after resuscitation, using PVC sponges

2   to collect wound fluid and cells. L-Arginine administration was 1 gm/kg/day intraperito-

3   neal in three divided doses, compared with saline controls. The L-arginine groups

4   showed increased OHP and wound breaking strength as compared with both shocked

5   and unshocked controls. Procollagen I and III mRNA expression was also enhanced by

6   L-arginine administration.[12]

7   Anaya-Prado et al, in a study of nitric oxide, also administered L-arginine at a

8   dose of 300 mg/kg.[13]   They compared Ringer's lactate (RL), alone, and with L-arginine,

9   sodium nitroprusside, or l-N^6-(1-iminoethyl) lysine (L-NIL, an iNOS inhibitor).   Blood

10 pressure recovery was improved in all three experimental groups relative to lactated

11 Ringer's controls. L-Arginine, nitroprusside, and L-NIL all showed a small, but non-

12 significant, improvement in survival as compared with controls. Of greatest significance,

13 all three treatment groups showed less liver necrosis, congestion, and vacuolization,

14 aspartate aminotransferase (AST), and alanine aminotransferase (ALT) relative to con-

15 trols. All treatments were characterized by decreases in levels of circulating cytokines.

16 The only cytokine elevated in the L-arginine group was IL-1β, and even that was re-

17 duced compared with controls and with the other treatment groups.

18   Other potential benefits of L-arginine have been pursued in various experimental

19 protocols. Preissler et al, for example, carried out experimental lung transplantation;

20 perfusing the excised lung with L-arginine.[14]   They had previously noted that transplant-

21 ing a lung from a donor animal subjected to hemorrhagic shock was associated with

22 poor functioning of the transplanted lung.   Perfusion of the isolated organ with L-

23 arginine prior to transplantation partially reversed the damage to the transplanted lung

1   produced by prior shock. They postulated that this was due to the amelioration of the

2   ischemia-reperfusion injury produced by the "double hit" of shock followed by excision of

3   the lung.

4   Findings from the present study allow the potential mechanism for the observed

5   protective effects of L-arginine in promoting survival from hemorrhagic shock to be con-

6   sidered in a somewhat different light.   In this respect results from previous studies have

7   established that L-arginine is a substrate for iNOS, and any exogenous L-arginine pro-

8   vided to an experimental animal will likely be converted, at least in part, to NO.   How-

9   ever, based upon the results of the present studies, it is quite clear that L-arginine has

10   considerably more widespread metabolic effects than simply allowing the generation of

11   nitric oxide. As perhaps might be expected from an amino acid used in every cell in the

12   body, it influences many biologic processes. Our data support the conclusion that L-

13   arginine supplementation can rather profoundly influence the level of gene expression

14   of a relatively large number of metabolic, inflammatory and acute phase genes. Even

15   confining the analysis to the signaling pathways shown in Table IV, the effects are mul-

16   tiple and widespread.

17   Of interest, the findings from the present study showed little evidence for an ef-

18   fect of L-arginine on blood pressure, while confirming the findings of Arora et al in the

19   swine model that L-arginine can improve survival following hemorrhagic shock. This

20   strongly suggests that the effect on blood pressure is not essential to the improvement

21   in survival. On the other hand, the effects on the liver found by Anaya-Prada et al may

22   well be important. It is clear from our data that the hepatic fibrosis and hepatic stellate

23   cell activation pathway were significantly affected by L-arginine, and a number of other

1 affected pathways are found in hepatocytes or in Kupffer cells of the liver. (Table III) In

2 studies of other agents, we have found that recovery of adenosine triphosphate in the

3 liver is prolonged following shock in the rat, requiring up to 48 hours to return to normal

4 levels. We and others have noted that shock is associated with a dramatic decrease in

5 cellular ATP levels in the liver.[15, 16] With at least three other agents, we have found that

6 improvement in survival and/or decreases in cytokine production are associated with

7 rapid recovery of liver ATP.[17-21] It appears that a number of interventions that can im-

8 prove outcome from shock also have the beneficial effect of enhancing recovery of liver

9 ATP. Liver ATP, then, may be a marker of recovery rather than a primary cause for it.

10 There is clinical evidence that arginine deficiency may be deleterious in septic

11 shock. A recent study by Gough, et al, found that arginine deficiency as measured by

12 the ration of arginine to dimethylarginines was predictive of mortality in patients with

13 sepsis.[22] A meta-analysis by Davis and Anstey showed that plasma arginine concen-

14 tration was markedly decreased in sesis.[23] Use of arginine has been advocated in sep-

15 tic shock, but with mixed results.[24-25] While the question remains open, current recom-

16 mendations are that arginine should be used only with caution in septic patients.[26] A

17 recent meta-analysis by Drover et al, however, found that the use of L-arginine-

18 supplemented diets in the peri-operative patient decreased both length of stay and inci-

19 dence of post-operative infections.[27] Little work has been done to study L-arginine sup-

20 plementation in patients with hemorrhagic shock or with trauma. Some early papers

21 showed encouraging results using L-arginine as one component of a multi-component

22 enteral "immunonutrition" supplement package, but the use of several components in

23 such studies makes it difficult to determine the effects of L-arginine by itself.[28-30]

1

2 **Conclusions**

3   The findings of the present study have confirmed and extended previous results

4 by others, showing that L-arginine administered with standards resuscitation fluids dur-

5 ing resuscitation from hemorrhagic shock can positively influence survival. The im-

6 provement in blood pressure noted by some other investigators was not observed, sup-

7 porting the conclusion that the mechanism involved in the protective effects of L-

8 arginine are more complex than simple hemodynamic improvement. The results of the

9 microarray analyses of gene response in circulating white blood cells provide further ev-

10 idence that the effects of L-arginine are widespread. The evidence supports the hy-

11 pothesis that L-arginine has a broad effect on a number of different metabolic and sig-

12 naling pathways in the body at the level of gene regulation. Additional work will be re-

13 quired to establish the underlying mechanisms by which L-arginine may make a critical

14 difference in positively affecting survival from shock. However, based on the results of

15 this study and those of other investigators, the use of L-arginine as an adjunct to resus-

16 citation from hemorrhagic shock continue to be considered seriously for clinical applica-

17 tion.

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1 **Table I: Survival**

Survival with or without L-Arginine in Resuscitation		
	Control	**Arginine**
Rat weight (gram)	286.70±18.02	294.40±21.50
Cannulating Time (minutes)	6.35±0.58	6.80±0.76
Bleeding (ml)	9.32±1.39	10.06±1.56
***Survival < 24 hours**	9/20*	16/20*
Survival > 72 hours	6/20	12/20
Shock: MAP = 20±4 mmHg, 90 minutes		
Resuscitated over 30 minutes with Ringer's lactate 21ml/kg) with and without L-arginine (300mg/kg) intravenously		
Data expressed as mean ± SD. *P< 0.05 (control *vs* Arginine).		

2

3

1   **Table II: Gene Activation following Shock and Resuscitation**

Experimental group	Genes upregu-lated	Genes down-regulated
Sham at 30 minutes vs sham baseline	4993	7178
Shock at 30 minutes vs shock baseline	4758	7579
Shock + LR at 30 minutes vs shock + LR baseline	5554	7510
Shock + LR + L-arginine at 30 minutes vs baseline	6143	6325

2

3

1 **Table III: Canonical pathways most differentially affected by arginine**

Ten canonical pathways most affected by arginine (Arranged in order of p-score for gene activation after L-arginine treatment)	
30 minutes	**4 hours**
cAMP-mediated Signaling	Hepatic fibrosis and hepatic stellate cell activation
G-protein Coupled Receptor Signaling	Wnd/β-catenin Signaling
VDR/RXR Activation	cAMP-mediated Signaling
Hepatic Fibrosis / Hepatic stellate cell Activation	Acute Phase Response Signaling
Glutamate Receptor Signaling	Glycerolipid Metabolism
Wnt/β-catenin Signaling	TGF-β Signaling
LXR/RXR Activation	Nitrogen Metabolism
Cardiac β-adrenergic Signaling	Glutamine Receptor Signaling
Androgen and Estrogen Metabolism	Cardiac β-adrenergic Signaling
Inositol Metabolism	Pentose and Glucuronate Interconversions

2

1 **Table IV: Changes seen in several canonical pathways relating to specific cytokines, comparing resuscitation**
2 **with L-arginine plus Ringer's lactate, versus Ringer's lactate alone (control).**
3
4 Key to canonical pathways: APR - Acute Phase Response Pathway; HC – Hepatic Cholestasi; HF – Hepatic Fibrosis and
5 Stellate Cell Activation

Cytokine	Gene	Protein	Control	Arginine	Pathway
IL-1	IL1R1	IL1 receptor	Up-regulated	Down-regulated	APR
IL-1	IL1RAP	IL1 receptor accessory protein	Down-regulated	Up-regulated	APR
IL-1	MYD88	Myeloid depression primary response	Down-regulated	Up-regulated	APR, HC
IL-1, TNF-α	JNK ½	JNK	Down-regulated	Up-regulated	APR
IL-1, TNF-α	MKK 4/7	MKK	Down-regulated	Up-regulated	APR
IL-1, TNF-α	IκB	IκB	Up-regulated	Neutral	APR
IL-4	IL-4R	Interleukin 4 receptor	Down-regulated	Up-reaulated	
IL-6	SHP2	Tyrosine phosphatase	Up-regulated	Down-regulated	APR
IL-6	JAK2	Janus kinase 2	Dow-regulated	Up-regulated	APR
IL-6	PDK1	Phosphoinisotol dependent kinase	Down-regulated	Up-regulated	APR
IL-6	cRAF	Murine leukemia viral homolog onco-gene	Down-regulated	Up-regulated	APR
IL-6	ERK ½	MAP kinase	Down-regulated	Neutral	APR
IL-6	Elk1	Member ETS oncogene family	Down-regulated	Up-regulated	APR
TNF-α	TTR	Transthyretin	Up-regulated	Down-regulated	APR
TNF-α	TLR4	Toll-like receptor 4	Down-regulated	Up-regulated	HC
Growth factors	PDGF-α	Platelet-derived growth factor α	Up-regulated	Down-regulated	HF
Growth factors	PDGFR-α	Platelet derived growth factor receptor α	Down-regulated	Up-regulated	HF
Growth factors	PDGFR-β	Platelet-derived growth factor receptor β	Up-regulated	Down-regulated	HF
Growth	LEPR	Leptin receptor	Up-regulated	Down-regulated	HF

factors					
Growth factors	FGFR1	Fibroblast growth factor receptor 1	Up-regulated	Down-regulated	HF
Growth factors	FGFR2	Fibroblast growth factor receptor 2	Up-regulated	Down-regulated	HF
Growth factors	IFBP5	Insulin-like growth factor binding protein	Up-regulated	Down-regulated	HF
Growth factors	CSF1	Colony stimulating factor 1	Neutral	Down-regulated	HF
NOS	AChR	Cholinergic receptor, muscarinic 1	Up-regulated	Down-regulated	NOS
NOS	PLN	Phospholanban	Up-regulated	Down-regulated	NOS
:NOS	ARG2	Arginase, type 2	Down-regulated	Neutral	LXR
Other	β-catenin	β-Catenin (transcription regulator)	Down-regulated	Up-regulated	WNT
Other	GRM5	Glutamate receptor, metabolomic 5	Up-regulated	Down-regulated	GLUT
	GRIP	Glutamate receptor interacting protein 1	Up-regulated	Down-regulated	GLUT

1

2

1 **Table V: Signature genes modified both in response to shock and additionally in**
2 **response to therapy with L-arginine**
3
4 *Gene   Protein encoded by the gene and its function*
5
6 ALOX5   Arachidonate 5-lipoxygenase (cytoplasmic enzyme)
7 B3GALNT1   Beta 1,3-N-acetyl/galactosaminyltransferase 2, membrane-bound
8 CXCL3   Chemokine (C-X-C motif) ligand 3, cytokine
9 CXCL11   Chemokine (C-X-C motif) ligand 11, cytokine
10 F3   Coagulation factor III, membrane receptor
11 GRID2   Glutamate receptor, ionotrophic, delta 2
12 GRM5   Glutamate membrane receptor
13 HSPA1B   Heat shock 70kDa protein 1b (chaperone protein)
14 HSPBAP   HSPB (heat shock 27kDa) associated protein 1 (chaperone protein)
15 IL1A   Interleukin-1, alpha, cytokine
16 IL1R2   Interleukin-1 receptor type II, membrane receptor
17 IL6   Interleukin-6, Cytokine
18 MSR1   Macrophage scavenger receptor 1, membrane receptor
19 NOS2A   Nitric oxide synthase 2A (inducible)
20 NLGN1   Neuroligin 1, membrane receptor
21 NPY1R   Neuropeptide Y receptor Y1, G-protein coupled membrane receptor
22 NR4A3   Nuclear receptor,steroid-thyroid hormone receptor superfamily
23 PLAUR   Plasminogen activator, urokinase receptor
24 RGS1   Regulator of G-protein signaling 1, membrane receptor component
25 ST3GAL3   ST3 beta-galactoside alpha-2,3-sialyltransferase 3, cytoplasmic enzyme
26 TNF   Tumor necrosis factor, TNF superfamily
27
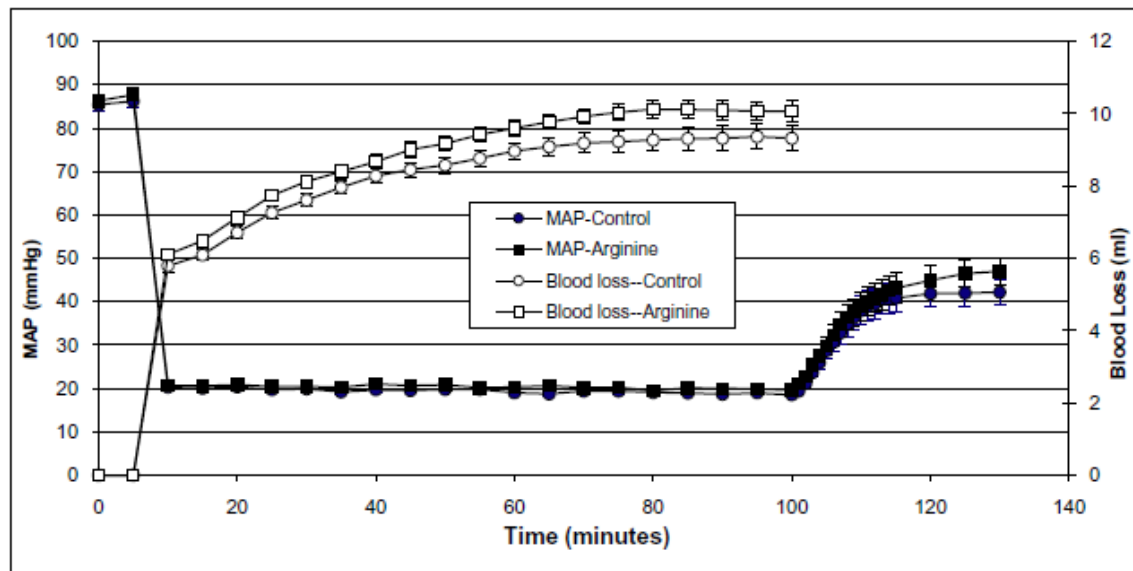
1 **Figure 1**

2 Schematic of the shock preparation for survival studies. There was a 10-minute equili-

3 bration period, a 90 minute shock period, and a 30 minute resuscitation period, after

4 which the cannulas were removed, and the animals allowed to recover from anesthesia.

5 There was a very small hemodynamic difference between the two groups (5 mm Hg), to

6 the benefit of the L-arginine group, as measured at the end of resuscitation. As well,

7 the L-arginine required slightly more blood to be withdrawn during the shock period to

8 maintain the blood pressure at 20 mmHg.

9

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Prefeeding With ω-3 Fatty Acids Suppresses Inflammation Following Hemorrhagic Shock

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What is This?

Original Communication

Prefeeding With ω-3 Fatty Acids Suppresses Inflammation Following Hemorrhagic Shock

Rongjie Yang, MD[1]; William S. Harris, PhD[2];
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Background: Hemorrhagic shock followed by resuscitation stimulates an inflammatory response. This study tests the hypothesis that prefeeding with fish oil rich in ω-3 fatty acids (FAs) will attenuate that response. *Methods:* Male Sprague-Dawley rats (n = 60; 350 ± 30 g) were randomly but unequally assigned to 3 groups: sham (n = 12), control (n = 24), and fish oil (n = 24). In the fish oil group, rat chow was supplemented with fish oil (600 mg/kg/d, 25% ω-3 FA). Control and sham group diets were supplemented with corn oil. Under fluothane, hemorrhagic shock was induced, and arterial pressure was maintained at 25 to 30 mm Hg for 30 minutes. Resuscitation was carried out by giving 21 mL/kg lactated Ringer's solution and returning shed blood to the animal. Half of each group was killed at 30 minutes and at 4 hours postresuscitation. Liver samples were assayed for indicators of inflammation and heat shock protein 25 (Hsp25). Lung edema was measured. *Results:* All animals survived. At 30 minutes postresuscitation, expression of mRNA for inducible nitric oxide synthase (iNOS) was significantly elevated in the control group but normal in the fish oil group. At 4 hours, expression of mRNA for Hsp25 was significantly increased in the fish oil group. Lung edema index was significantly lower in the fish oil group than in either sham or control groups. *Conclusions:* Fish oil prefeeding in a rodent model of hemorrhagic shock was associated with increased liver mRNA expression of Hsp25, reduced liver mRNA expression of iNOS, and decreased lung edema. These findings support the validity of the study hypothesis. (*JPEN J Parenter Enteral Nutr.* 2010;34:496-502)

Keywords: fish oil; hemorrhagic shock; heat shock protein 25; interleukin-1beta; inducible nitric oxide synthase; mRNA

Hemorrhagic shock followed by resuscitation is known to stimulate a systemic inflammatory response. Preventing the catastrophic consequences of hemorrhagic shock remains an unsolved problem in clinical treatment of trauma. Previous studies from our group have shown that a number of inflammation-related genes are overexpressed in hepatic tissues following hemorrhagic shock and resuscitation,[1,2] and these responses precede tissue apoptosis and resulting tissue damage.

Our hypothesis was that prefeeding experimental animals with fish oil rich in ω-3 fatty acids, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), would help to minimize the postshock inflammatory responses. These fatty acids have been reported to modulate inflammation and have been used clinically for that purpose.[3,4] In this study, fish oil rich in EPA and DHA was prefed to animals that were then subjected to sublethal hemorrhagic shock. Control animals were fed with ω-6 fatty acids.

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Materials and Methods

Nutrition Supplements and Prefeeding

Ten- to 12-week-old male Sprague-Dawley rats (n = 60; 350 ± 30 g, Charles River Laboratories, Wilmington, MA) were randomly assigned into a sham group (n = 12),

control group (n = 24), or fish oil group (n = 24), or then fed with appropriate diets. Animals were maintained in the Animal Care Center of the University of Missouri–Kansas City and were kept on a 12-hour light-dark cycle with free access to food and water. In the fish oil group, the rat chow diet was supplemented with commercial fish oil 600 mg/kg/d (25% DHA and EPA). This provided ω-3 fatty acids equivalent to about 2% of the daily energy requirement. In the sham and control groups, an equivalent amount of corn oil was added to the rat chow. Diets were stored in the dark at a cold room temperature of 4°C until fed, animals were allowed access to food ad libitum, and food remaining in the cage from the previous day was discarded. All diet preparations were done by the supplier (Dyets, Inc, Bethlehem, PA). Animals were subjected to hemorrhagic shock after 4 weeks of prefeeding.

Hemorrhagic Shock Model

All rats were anesthetized with isoflurane (Baxter Healthcare Corporation, Deerfield, IL) using an anesthesia vaporizer (SurgiVet, Inc, Waukesha, WI) with 100% oxygen while breathing spontaneously. Isoflurane was adjusted from 3% to 1% after induction and then maintained at 1% until the conclusion of the experiment. The femoral artery and femoral vein were cannulated through a short groin incision.[1,2] Heparin (200 U/kg) was administered following cannulation. Hemorrhagic shock was induced by removing blood slowly through the left femoral arterial cannula. The mean arterial pressure (MAP) was maintained at 25 to 30 mm Hg for 30 minutes in the control and ω-3 fatty acid groups with periodic withdrawal of additional blood. MAP was measured continuously using a blood pressure monitor (Micro-Med, Louisville, KY). Resuscitation was carried out by giving 21 mL/kg lactated Ringer's solution and returning the shed blood to the animal at 0.5 mL/min. The animals in the sham group received anesthesia and surgical preparation and were then kept anesthetized for an equivalent length of time to that of the control and experimental groups but were not subjected to shock. At the end of 30 minutes of shock, the cannulas were withdrawn and the artery and vein ligated. In half the animals, liver samples were collected for mRNA at 30 minutes postresuscitation (n = 30; sham = 6, control = 12, and ω-3 = 12). The rest of the animals (n = 30) were awakened and placed in a clean cage. They were reanesthetized, and liver samples were collected for mRNA at 4 hours postresuscitation. All animals were killed while anesthetized using aortic transection.

Real-Time Polymerase Chain Reaction

Liver tissue was collected as noted above and gently homogenized. Isolation of mRNA was done using a commercial kit (QIAGEN RNA/DNA Mini Kit; QIAGEN, Inc; Valencia, CA). The oligonucleotides used are shown in Table 1.

Table 1.   Oligonucleotide Primers for RT-PCR

Protein	Direction	Primer Sequence
IL-1β	Forward	5′-GGATGATGACGACCTGC-3′
	Reverse	5′-CTTGTTCCGTTATGTTCTG-3′
iNOS	Forward	5′-GGAGAGATTTTTCACGACACCC-3′
	Reverse	5′-CCATGCATAATTTGGACTTGCA-3′
Hsp25	Forward	5′-TGTCAGAGATCCGACAGACG-3′
	Reverse	5′-GCCTTCCTTGGTCTTCACTG-3′
IL-6	Forward	5′-CAAAGCCAGAGTCCATTCAGAGC-3′
	Reverse	5′-GGTCCTTAGCCACTCCTTCTGT-3′
IL-8	Forward	5′-GACTGTTGTGGCCCGTGAG-3′
	Reverse	5′-CCGTCAAGCTCTGGATGTTCT-3′
IL-10	Forward	5′-AAAGCAAGGCAGTGGAGCAG-3′
	Reverse	5′ TCAAACTCATTCATGGCCTTGT-3′
β-actin	Forward	5′-TTGCTGACAGGATCGAGAAG-3′
	Reverse	5′-TAGAGCCACCAATCCACACA-3′

Hsp25, heat shock protein 25; IL, interleukin; iNOS, inducible nitric oxide synthase; RT-PCR, reverse transcription–polymerase chain reaction.

Reverse transcription–polymerase chain reaction (RT-PCR) was carried out using the 2-step method. First, cDNA was synthesized using a commercial kit (SuperScript III First Strand Synthesis System; Invitrogen, Carlsbad, CA). Quantitative PCR was then carried out using the Opticon 2 real-time PCR detection system (Bio-Rad Laboratories, Waltham, MA). Oligonucleotides were prepared as specified in Table 1 and were ordered from a commercial supplier (Sigma-Genosys, Woodlands, TX). The resulting data were analyzed using the 2 exp(–ΔΔDDT) approach to allow comparison among the 3 groups.

Fatty Acid Analysis

To verify that the dietary changes resulted in the incorporation of significant amounts of DHA and EPA into membrane lipids, fatty acid analysis was carried out in red blood cells (RBCs) and in liver cells. RBCs were separated from the plasma by centrifugation of blood at 1,500 *g* for 20 minutes at 4°C. RBCs were stored at –70°C until analysis. Packed RBCs were thawed, and 50 μL was dried at 45°C under nitrogen. Thereafter, the samples were directly methylated by the addition of 0.5 mL 14% boron trifluoride methanol (Sigma, St. Louis, MO) and then heated at 100°C for 10 minutes. This generated fatty acid methyl esters (FAMEs) from the glycerophospholipids in the RBC membranes.[5] After cooling, 1.0 mL water and 1.0 mL hexane containing 50 mg/L butylated hydroxytoluene (BHT, an antioxidant; Sigma) were added. Following manual shaking for 30 seconds, the samples were centrifuged for 3 minutes at 1,500 *g* at room temperature. The hexane layer was collected, the solvent was evaporated at 45°C under nitrogen, and the FAMEs thus generated were reconstituted with 50 μL hexane and analyzed by gas chromatography (GC) with flame ionization detection.

Liver samples were first homogenized and the cell pellet isolated by centrifugation and frozen until analysis. Total liver lipids were extracted with methanol and methylene chloride (both containing 50 mg/L BHT) as described.[6] The organic phase was collected and the solvent evaporated under nitrogen in a 45°C water bath. The samples were methylated as described.[5] The samples were analyzed by GC (GC-14A; Shimadzu, Columbia, MD) using a fused silica capillary column (SP-2560, 100 m; Supelco, Bellefonte, PA). FAMEs are reported as percent of total and were identified by comparison with known standards.

Cytokine Analysis

Tumor necrosis factor α (TNF-α) and interleukin (IL)-6 were analyzed using the Quantikine assay kit (R&D Systems, Minneapolis, MN). This assay used a quantitative sandwich enzyme-linked immunosorbent assay (ELISA). The monoclonal antibody was precoated onto a microplate. After placing the samples, controls, and standards into the wells, available mouse IL-6 was bound by the antibody. Excess was washed, and a second enzyme-linked polyclonal antibody was added. After a second wash, a solution of enzyme substrate was added. This was then converted to a yellow chromogen with an acidic stop solution, and samples were measured spectrophotometrically. Actual amounts were calculated from a standard curve.

Nitrite Analysis

Nitric oxide activity was estimated by measuring nitrite, using a Greiss reagent system (Promega, Madison, WI). Samples, standards, and controls were placed in a 96-well plate. Sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) was added. After a 10-minute incubation, N-(1-naphthyl)-ethylenediamine dihydrochloride was added. After another 10 minutes, the absorbance 520 to 550 nm was measured on a plate reader. Concentrations of nitrite were read from the standard curve.

Lung Tissue Edema Index

After removal, the lungs were patted dry with a tissue and weighed. They were then dried overnight in an oven and reweighed. The lung edema index was calculated as the ratio between the wet weight and the dry weight.

Institutional Approval

All animal care and experimental procedures were carried out according to the guidelines of the Laboratory Animal Center of the University of Missouri–Kansas City, which is a facility accredited by the American Association for the Accreditation of Laboratory Animal Care. The experimental protocols under which this work was carried out were approved by the Institutional Animal Care and Use Committee of the University of Missouri–Kansas City.

Table 2.   Hemorrhagic Shock Parameters[a]

	Sham	Control	Fish Oil	Control vs Fish Oil
Weight, g	341 ± 4	349 ± 6	347 ± 6	NS
Cannulation time, min	7.5 ± 0.4	8.2 ± 0.3	8.1 ± 0.4	NS
Blood removed, mL	0	9.2 ± 0.3	9.6 ± 0.2	NS

NS, not significant.
[a]Data are given as mean ± standard error of the mean.

Statistical Analysis

Group sizes (n = 12) were determined using power analysis (PASS; NCSS, Kaysville, UT). A group size of 12 was determined to provide an 80% chance of detecting a 2:1 variation in the levels of cytokines, or a 2-fold increase in the mRNA measured by RT-PCR using the 2^DDCt method. Data analysis was carried out with the Aspin-Welch unequal-variance *t* test for experimental vs control groups, using NCSS. The Benjamini-Hochberg method was used to minimize the false discovery rate for multiple *t* tests.[7] Data results are presented as mean ± standard error of the mean. A significance level of .05 was used for all analyses.

Results

Shock Preparation

All animals survived shock and resuscitation in all groups. Among the groups, there was no difference in the return of blood pressure to baseline following shock and resuscitation (Table 2). Except for the sham group, all animals were maintained at 25 to 30 mm Hg for 30 minutes by periodic withdrawal of blood, without having to add blood back. There were no detectable differences among the groups in terms of weight, cannulation time, or amount of blood withdrawn (except again for the sham group; Table 2).

Lipid Analysis

Levels of DHA and EPA were found to be significantly elevated in RBC membrane lipid preparations as compared with controls in both tissue types, whereas levels of

Table 3. Lipid Content of Red Blood Cell Membranes[a]

Group	EPA (C20:5 ω-3)		DHA (C22:6 ω-3)		AA (C20:4 ω-3)	
	Mean	SD	Mean	SD	Mean	SD
Control (n = 24)	0.19	0.13	1.91	0.41	21.09	2.37
Fish oil (n = 24)	1.82	0.27	4.88	0.94	16.94	1.46

AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SD, standard deviation.
[a]$P < .001$ for all groups.

Table 4. Lipid Content of Cell Membranes Extracted From Liver Homogenate[a]

Group	EPA (C20:5 ω-3)		DHA (C22:6 ω-3)		AA (C20:4 ω-3)	
	Mean	SD	Mean	SD	Mean	SD
Control (n = 24)	0.05	0.01	4.18	2.15	21.08	3.40
Fish oil (n = 24)	2.31	0.67	13.04	2.25	13.95	2.63

AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SD, standard deviation.
[a]$P < .001$ for all groups.

arachidonic acid (AA) were correspondingly reduced (Tables 3 and 4). Thus, we concluded that the 4-week time period selected for these studies was sufficient for incorporation of significant amounts of ω-3 fatty acids into lipid membranes.

TNF-α and IL-1β

TNF-α was measured 30 minutes after shock and resuscitation because it is known to be characteristic of an early response to hemorrhagic shock. As expected, TNF-α was markedly elevated in both control and fish oil groups but not in the sham group, reflecting the effect of shock (Table 5). There was no significant difference between the control and fish oil groups.

IL-6 was measured 4 hours postresuscitation because it characteristically has a later response. Interestingly, levels of this cytokine were elevated in sham animals as well, with no significant difference between the control and fish oil groups (Table 5).

Nitric Oxide Production

The nitrite concentrations, reflective of the presence of inducible nitric oxide synthase, were elevated at 30 minutes in all 3 groups, and the concentrations were reduced to about one quarter of peak values at 4 hours (Table 5). There were no significant detectable differences between control and fish oil groups.

Lung Edema Index

The lung edema index was measured in tissue samples collected from the animals at 4 hours (Table 5; Figure 1). It was significantly lower in the fish oil group than in the control group. It was also elevated in the sham group, which was fed the same diet as the control group.

mRNA for IL-1β, IL-6, IL-8, IL-10, Hsp25

The levels of mRNA for cytokines were measured in hepatic tissues at 4 hours (Table 6). Expression of mRNA for IL-1β was increased in the control group as compared with the fish oil group. The levels of expression of mRNA for IL-6 and IL-8 were reduced in both control and fish oil groups as compared with the unshocked sham group, with no significant difference between the control and fish oil groups. At 4 hours postresuscitation, expression of mRNA for heat shock protein 25 (Hsp25) was increased in the fish oil group relative to the control group (Figure 2).

iNOS mRNA

The levels of mRNA for inducible nitric oxide synthase (iNOS) were measured at both 30 minutes and 4 hours postresuscitation (Table 7; Figure 3). At 30 minutes, the fish oil group showed a significant decrease in iNOS message as compared with the control group. At 4 hours, there was no significant difference, and variability was high. It should be noted that although mRNA for iNOS was much lower in the fish oil group, the nitrite concentration (see Nitric Oxide Production; Table 5) was no different than in the control group at 30 minutes.

Discussion

Daily dietary supplementation with ω-3 fatty acids has been well established as a method to achieve a reduction in risk of cardiovascular disease and is used experimentally for alleviation of certain acute systemic inflammatory diseases such as asthma, chronic obstructive pulmonary disease, and inflammatory bowel disease.[8] There have been a number of studies that used ω-3 fatty acids in critically ill patients. A 2001 meta-analysis of 22 published reports indicated that the overall effect of fish oil in immunomodulation was to reduce complications but not mortality.[9] A very limited meta-analysis published in 2008, which included only 3 reports in the literature on the use of EPA and DHA in acute respiratory distress syndrome, showed a strong beneficial effect on mortality and other outcome measures.[10] Clinical use of ω-3 fatty acids in the systemic inflammatory response syndrome remains very much an open question, which in part prompted the present study.

Table 5.　Results of Cytokine Protein Measurements (pg/mL Plasma) and Lung Edema Index (Wet/Dry Weight)[a]

	30 Minutes Postshock			4 Hours Postshock		
Quantity Measured	Sham	Control	Fish Oil	Sham	Control	Fish Oil
TNF-α	<1	1,220 ± 445	1,605 ± 657	—	—	—
IL-6	—	—	—	589 ± 26	568 ± 15	567 ± 13
Nitric oxide	190 ± 11	166 ± 18	189 ± 20	42.6 ± 1.3	42.6 ± 1.2	44.8 ± 1.9
Lung edema index	—	—	—	5.14 ± 0.18	5.28 ± 0.28[b]	4.69 ± 0.14[b]

IL, interleukin; TNF-α, tumor necrosis factor α.
[a]Data are given as mean ± standard error of the mean.
[b]$P < .05$ (*t* test, control group vs fish oil group).
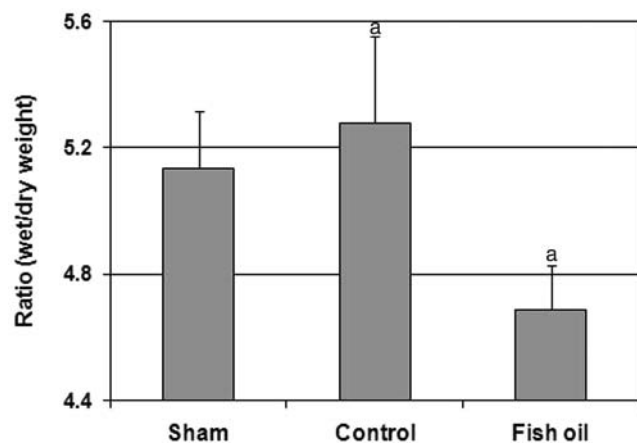
Figure 1.　Lung edema index, showing less lung water accumulation in the fish oil group, fed ω-3 fatty acids, than in the control group, fed ω-6 lipids. [a]$P < .05$, control group vs fish oil group.
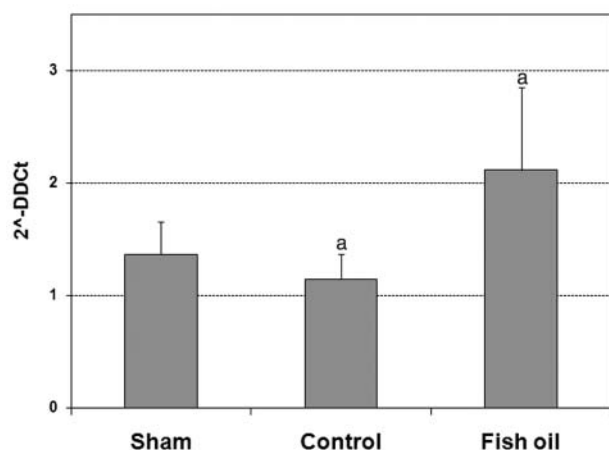
Figure 2.　Expression of mRNA for heat shock protein 25 (Hsp25), relative to β-actin, calculated using the 2^DDCt method. Indicates that mRNA for this chaperone protein was expressed more strongly in the fish oil group, fed ω-3 fatty acids, than in the control group, fed ω-6 lipids. [a]$P < .05$, control group vs fish oil group.

Table 6.　Results of RT-PCR Analysis for Cytokines and Hsp25, 4 Hours Postshock[a]

	4 Hours Postshock		
Quantity Measured[b]	Sham	Control	Fish Oil
IL-1β mRNA	1.34 ± 0.56	3.39 ± 1.56	1.83 ± 0.75
IL-6 mRNA	1.36 ± 0.92	0.22 ± 0.08	0.36 ± 0.11
IL-8 mRNA	1.17 ± 0.61	0.48 ± 0.12	0.53 ± 0.28
IL-10 mRNA	1.05 ± 0.32	2.82 ± 1.20	2.18 ± 0.82
Hsp25 mRNA	1.36 ± 0.29	1.14 ± 0.22*	2.11 ± 0.74[c]

Hsp25, heat shock protein 25; IL, interleukin; RT-PCR, reverse transcription–polymerase chain reaction; TNF-α, tumor necrosis factor α.
[a]Data are given as mean ± standard error of the mean.
[b]Relative to β-actin, calculated using the 2^DDCt method.
[c]$P < .05$ (*t* test, control group vs fish oil group).

Table 7.　Results of RT-PCR Analysis for iNOS[a]

Quantity Measured[b]	Time	Sham	Control	Fish Oil
iNOS mRNA	30 minutes	2.08 ± 1.47	1.42 ± 0.71[c]	0.25 ± 0.13[c]
iNOS mRNA	4 hours	1.05 ± 0.32	6.39 ± 3.97	12.3 ± 9.37

iNOS, inducible nitric oxide synthase; RT-PCR, reverse transcription–polymerase chain reaction.
[a]Data are given as mean ± standard error of the mean.
[b]Relative to β-actin, calculated using the 2^DDCt method.
[c]$P < .05$ (*t* test, control group vs fish oil group).

There is evidence that ω-3 fatty acids can be incorporated into cell membranes within the lung and that this may have a beneficial effect. Breil et al[11] used a 3-hour infusion of fish oil emulsion into isolated rabbit lungs and showed that even a brief infusion resulted in lowered vascular reactivity and decreased edema formation in response to a pharmacologic stimulus, as compared with controls perfused with a soybean oil emulsion. In an experimental study of endotoxin shock, Mancuso et al[12] found that administration of fish oil and/or borage oil reduced pulmonary vascular permeability. Both of these groups[11,12] attributed the changes to production of lipid
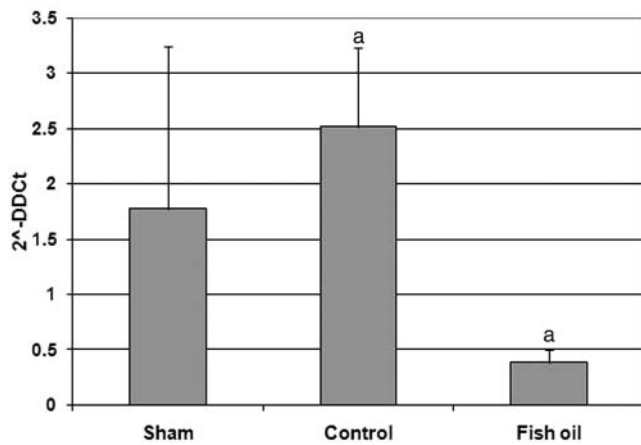
Figure 3. Expression of mRNA for inducible nitric oxide synthase, relative to β-actin, calculated using 2^DDCt method. Indicates that this was expressed less strongly in the fish oil group, fed ω-3 fatty acids, than in the control group, fed ω-6 lipids. [a]*P* < .05, control group vs fish oil group.

intermediaries of ω-3 origin. Pierre et al[13] studied ω-3 fatty acid administration in a rat model of sepsis from *Pseudomonas aeruginosa*, finding improved survival, which they attributed to multiple factors.

Few investigators have examined the potential protective contribution of fish oil in experimental shock. Ertel et al[14] prefed mice with ω-3 fatty acids for 3 weeks, subjected them to hemorrhagic shock and resuscitation, and harvested peritoneal macrophages and splenocytes at 24 hours postresuscitation. They found that fish oil prevented an increase in levels of circulating prostaglandin E_2 (PGE_2) release seen in mice fed with corn oil or safflower oil. Dietary fish oil also prevented a reduction in antigen presentation capacity and consequent suppression of IL-1 release. Our own studies reported here were carried out with liver cells at 4 hours and have provided evidence that prefeeding with fish oil had a more limited effect, suppressing early mRNA expression for only a few components of the inflammatory response. This suggests either that the time course may be important or that liver cells may react differently from macrophages.

Additional studies in this area have been done using sepsis models. Oz et al[15] showed that 6 days of a fish oil diet could protect rats from the hepatic damage associated with lipopolysaccharide (LPS) administration. A modest reduction in circulating levels of interferon γ was observed at 4 hours after LPS administration, but no change was seen in either IL-6 or TNF-α.

Babcock et al[16] studied LPS-stimulated murine macrophage-like RAW 264.7 cells and showed that levels of phosphorylation of proteins in the mitogen-activated protein kinase (MAPK) pathway were significantly reduced by administration of ω-3 fatty acids. The reduction was associated with suppression of activation of the transcription factor activator protein (AP)–1. Novak et al[3] also studied RAW 264.7 cells stimulated with LPS and found that pretreatment with ω-3 fatty acids inhibited I κB phosphorylation and decreased nuclear factor κB (NF-κB) activation. There was a decrease in levels of TNF-α mRNA and protein. Lo et al[17] showed a similar finding for prefeeding with EPA alone.

Mishra et al[18] studied murine aortic endothelial cells, showing that "oxidized EPA" inhibited endothelial MCP-1 and IL-8 expression and that it inhibited NF-κB activation but not AP-1 activation. No specific oxidation product was identified. Other studies provided evidence that oxidized EPA is a strong activator of peroxisome proliferator-activated receptor α (PPAR-α), and the above effects were not seen in PPAR-α–deficient cells. Auto-oxidation of EPA occurs naturally, and the authors speculated that the oxidized compounds have anti-inflammatory properties acting through a PPAR-α–dependent mechanism.

As the data from the present study indicate, one of the constant results of feeding with ω-3 fatty acids is incorporation of EPA and DHA into cell membranes. Is it possible that the changed hydrophobic local lipid environment of the cell membrane affects receptor function on the cell surface? There is evidence that DHA and EPA can affect the characteristics of crucial areas of the cell membrane. A review by Ma et al[19] summarizes work done in several cell lines on the effect of ω-3 fatty acids on lipid rafts, substructures of the lipid membrane, sometimes characterized as detergent-resistant membrane fragments. The available evidence would strongly support the conclusion that these structures are critical in cell signaling, concentrating many of the receptors and signaling proteins. Switzer et al,[20] from the same institution, studied the effect of ω-3 fatty acids on T-cell membrane function and hypothesized that these fatty acids modulate T-cell function by altering composition of the lipid rafts. Schley et al[21] found that EPA and DHA altered the composition of lipid rafts in human breast cancer cells, specifically decreasing epidermal growth factor receptor (EGFR) activity and increasing phosphorylation of EGFR and MAPK. Chen et al[22] showed that DHA was incorporated into the lipid rafts of human retinal vascular endothelial cells and displaced cholesterol. Furthermore, this was associated with inhibition of TNF-α– and IL-1β–induced cellular adhesion molecule (CAM) expression and with TNF-α–induced NF-κB activation.

The "classic" explanation for the effects of ω-3 fatty acids on regulation of inflammatory host responses is that the administration of ω-3 fatty acids promotes the formation of eicosanoids, which are anti-inflammatory rather than proinflammatory.[22] Ingestion of fish oil (EPA and DHA) leads to decreased PGE_2, thromboxane A_2, and leukotriene B_4, with increases in thromboxane A_3, prostacyclin PGI_3, and leukotriene B_5. More generally, the leukotrienes, prostacyclins, thromboxanes, and prostaglandins derived from ω-3 fatty acids are less proinflammatory than the corresponding compounds derived

from ω-6 fatty acids. There are, however, powerful anti-inflammatory metabolites from the ω-6 fatty acid AA, and one cannot say with validity that "ω-6 are proinflammatory and ω-3 are not."[23]

The results from the present study support our hypothesis that prefeeding with ω-3 fatty acids can alter the inflammatory response to hemorrhagic shock. Prefeeding with a diet rich in ω-3 fatty acids, as compared with a diet rich in ω-6 fatty acids, clearly modulated the host response, as indicated by iNOS and Hsp25, following hemorrhagic shock. Elevation of Hsp25, as was observed, is associated with increased cytoprotection. Lung edema, an indicator of the systemic inflammatory reaction, was decreased. From the above discussion, elucidating the mechanism(s) responsible for this effect will be an important next step in this area of investigation.

Conclusions

Fish oil prefeeding in a rodent model of hemorrhagic shock was associated with reduced liver mRNA expression of iNOS, increased mRNA expression of Hsp25, and decreased lung edema. These findings support the hypothesis that supplementation with DHA and EPA can modulate the inflammatory reaction following hemorrhagic shock.

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Original Communication

Crocetin Reduces Activation of Hepatic Apoptotic Pathways and Improves Survival in Experimental Hemorrhagic Shock

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Background: Hemorrhagic shock results in cellular damage and cell death. A primary mechanism is cellular apoptosis from mitochondrial damage. This study demonstrated that administration of crocetin to experimental animals during resuscitation from shock significantly improved postshock survival and reduced apoptosis. Crocetin is a component of saffron and has long been used in traditional medicine in Asia. *Methods:* Male Sprague-Dawley rats (350 ± 30g) were randomly assigned to 1 of 4 groups of 8 animals. Hemorrhagic shock was induced by withdrawing blood until the mean arterial pressure was 35–40 mm Hg, and blood pressure was maintained at that level for 60 minutes with further withdrawals as needed. Resuscitation was carried out by administration of 21 mL/kg lactated Ringer's solution and return of shed blood, with or without concurrent administration of crocetin (2 mg/kg). Control animals were sham-treated with surgical preparation, without shock or resuscitation, and with and without crocetin. Rats were sacrificed 24 hours after completion of resuscitation. The extent of activation of hepatic apoptosis was established by measuring levels of hepatic cytosolic cytochrome c, caspase-3, and bcl-2. A separate group of 53 animals treated identically was used to assess survival. *Results:* Crocetin administration during resuscitation resulted in less extensive activation of hepatic apoptosis and significantly increased survival relative to controls. *Conclusions:* Crocetin administration to experimental animals during resuscitation post hemorrhage increased survival, at least in part by protecting the liver from activation of apoptotic cell death. This agent continues to show promise as a potential treatment strategy for hemorrhagic shock. (*JPEN J Parenter Enteral Nutr.* 2011;35:107-113)

Keywords: inflammatory mediators; cytokines; resuscitation; cytochrome c; caspase-3; Bcl-2 protein; saffron; oxygen diffusion

Hemorrhagic shock, a major complication of trauma, is a relatively common and potentially critical medical problem for which optimal therapeutic interventions have yet to be identified. A primary cause of hemorrhage-associated problems is the development of extensive postresuscitation tissue injury mediated by inflammatory mediators, leading to cellular apoptosis.

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We and others earlier reported that in animals subjected to controlled hemorrhagic shock followed by resuscitation, extensive hepatic apoptosis can be readily detected within 24 hours. It is highly likely that this cellular apoptosis is at least one of the major mechanisms by which significant organ damage results during and following reperfusion leading to tissue injury.[1,2]

Crocetin has long been used in traditional Chinese medicine as an agent for the treatment for many human diseases, including gout, cancer, depression, constipation, and infections, among others. Structurally, crocetin has been shown to consist of a linear 16-carbon chain, of which 8 of the linkages are double bonds. There are also 4 attached internal methyl groups, and a carboxylic acid (—COOH) group is positioned at each end of the molecule. The molecular weight of this compound is 328 Da (Figure 1).
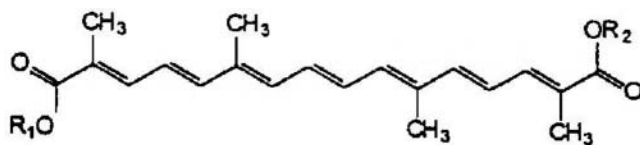
Figure 1. Structure of the crocetin molecule.

Present in many foods, crocetin is a major constituent of saffron and is responsible for the characteristic yellow color of that spice. Besides the crocus plant (*Crocus sativus L*), crocetin is found in a number of other relatively common plants and is commercially extracted from gardenia root (*Gardenia jasminoides Ellis*). It is commonly sold in the United States as an herbal supplement.

In terms of crocetin's fundamental mode of action in host tissues that might explain some of its therapeutic properties, crocetin was initially reported by Gainer and others to cause increases in oxygen diffusion in vitro and to improve tissue oxygenation post resuscitation in vivo.[3-7] However, the effects of this agent on actual survival were not evaluated in those studies. In our own earlier published studies, we showed that administration of crocetin to adult male rats during resuscitation following experimentally induced hemorrhagic shock improved the recovery of cellular adenosine triphosphate (ATP) in liver and resulted in significantly reduced expression of inflammation-related genes in a rat model of hemorrhagic shock and resuscitation.[8-10]

To better understand the underlying mechanisms by which crocetin can potentially modulate the host response to hemorrhagic shock, we evaluated the effect of crocetin administration on the level of postresuscitation hepatic apoptosis in a sublethal experimental model of hemorrhagic shock followed by resuscitation. In the studies reported here, we evaluated the extent of hepatic cellular apoptosis by quantitating levels of hepatic cytochrome c, caspase 3, and bcl-2 during the early period post shock (24 hours). In a separate group of rats, we monitored survival over 72 hours following treatment with crocetin at the time of resuscitation relative to control rats treated with resuscitation alone. Our hypothesis in this study was that crocetin administration would significantly reduce the levels of hepatic cellular apoptosis, which would correlate with an increased number of animals surviving relative to untreated controls.

Methods

Experimental Animal Hemorrhagic Shock Model

Sprague-Dawley male rats (n = 32, 350 ± 30 g; purchased from Charles River, Wilmington, MA) were randomly assigned to 4 groups, each group containing 8 animals:

1. Sham control group—anesthesia and surgical preparation, no hemorrhagic shock, no crocetin intervention
2. Sham crocetin group—anesthesia and surgical preparation, no hemorrhagic shock, crocetin administered
3. Control group—hemorrhagic shock, no crocetin intervention, resuscitated with lactated Ringer's solution (LR) alone
4. Crocetin group—hemorrhagic shock, resuscitated with LR and administered crocetin during resuscitation

All rats were anesthetized with 3% isoflurane (Baxter Healthcare Corporation, Deerfield, IL) with an anesthesia vaporizer (SurgiVet, Waukesha, WI) with 100% oxygen while breathing spontaneously. Isoflurane was adjusted from 3% to 1% after induction and then maintained at 1%–1.5% until the conclusion of the resuscitation.

After anesthetization, animals were covered with a sterile drape. The temperature under the drape, controlled by a lamp over the table, was maintained at approximately 28°C during the shock period and 36°C during the resuscitation period. After sterile skin preparation, a cervical incision was made. The left carotid artery and jugular vein were cannulated using PE50 polyethylene tubing (Becton Dickinson, Sparks, MD). All animals were anticoagulated with heparin (300 units/kg) through the venous cannula after the procedure was completed. A digital blood pressure analyzer (Micro-Med, Louisville, KY) was used to monitor pressure continuously. Hemorrhagic shock was initiated by withdrawing blood through the carotid arterial cannula over a 5-minute period, until the mean arterial pressure (MAP) stabilized at 35–40 mm Hg, MAP was maintained at that level with further withdrawals of blood, as required, for 60 minutes. MAP was monitored continuously and recorded every 5 minutes.

In all animals subjected to hemorrhagic shock, resuscitation was carried out by administration of LR, 21 mL/kg, followed by return of the shed blood to the animals previously subjected to hemorrhagic shock. Resuscitation fluid and blood were given over a total of 30 minutes. In the crocetin treatment group, crocetin (ICN Biomedicals, Aurora, OH), dissolved in normal saline (0.9%), was administered as a bolus of 2 mg/kg (1 mL/kg of solution, about 0.35 mL) at the time of resuscitation. The crocetin dosage used was determined on the basis of dose–response studies carried out in conjunction with previously reported studies.[8-10] As noted below, a larger dose was used following the longer period of shock used in the chronic study.

Control shock animals received an equivalent bolus of normal saline (1 mL/kg). For the sham control and sham crocetin groups, either normal saline or crocetin as above was given, without administration of LR.

All animals were allowed to recover from anesthesia. At 24 hours following resuscitation, a midline laparotomy

incision was made, and liver biopsies were taken. Samples were frozen immediately in liquid nitrogen and then stored in a refrigerator at –80°C for further studies. After sampling, animals were sacrificed by exsanguination.

Measurements of Apoptosis

Frozen tissue samples were thawed and gently homogenized, and particulate material was removed by centrifugation. The resulting liver cytosolic extracts were analyzed for markers indicative of relative levels of hepatic apoptosis and levels of cytochrome c, caspase-3, and bcl-2 quantified using enzyme-linked immunosorbent assays (ELISAs) or enzyme activity assays. All assays were compared to purified standards provided by the manufacturers run in parallel. Relative extent of apoptosis was measured using the Cell Death Detection ELISA-PLUS kit (Roche Applied Science, Indianapolis, IN). Cytochrome c was analyzed using a commercially available assay kit (MBL International, Woburn, MA). Caspase-3 was assayed using the Caspase-3 Protease Assay kit (BioSource International, Camarillo, CA). Bcl-2 was analyzed with a bcl-2 ELISA kit (Oncogene Research Products, San Diego, CA).

Animal Survival Studies

For survival studies, male Sprague-Dawley rats (350 ± 30 g, n = 53) were subjected to hemorrhagic shock using a protocol almost identical to that described above but with the difference that a mean arterial pressure of 25–30 mm Hg was maintained for 60 minutes. Two groups of rats were studied, 1 resuscitated with LR (21 mL/kg) alone and the other with LR (21 mL/kg) plus crocetin (4 mg/kg), with both groups receiving return of shed blood. Following recovery from the surgery, all animals were monitored until death or for 72 hours. The larger dose of crocetin was used to compensate for the longer period of shock, in an effort to maximize any potential contribution of the intervention on survival.

Statistical Analysis

Sample size for rats used in both the acute and chronic experiments was calculated using power analysis (PASS, NCSS, Kaysville, UT). For the acute survival experiments, a sample size of 8 was determined to provide a 98% chance of detecting a 2:1 variation in the levels of apoptosis and cytochrome c detected in hepatic extracts of the experimental group vs controls. For the longer term survival experiments, a sample size of 26 was estimated to provide an 87% chance of detecting a 2:1 difference in overall survival between the treated and control animals assuming an approximate control mortality of 80%.

Data analysis was carried out with Microsoft Excel (Microsoft, Redmond, OR) and with NCSS (NCSS, Kaysville, UT). Data results and graphs were expressed as mean ± standard error of the mean. Data were further analyzed using analysis of variance, Student's *t* test, and Kruskal-Wallis multiple-comparison *z*-value test (NCSS, Kaysville, UT). One-tailed analysis of variance was used to compare survival rates (Microsoft Excel). Survival data were further analyzed with Fisher's exact test. The significance level was .05 for all analyses.

Institutional Approval

All animal care and experimental procedures were carried out strictly in accordance with the Guidelines of the Laboratory Animal Center of the University of Missouri–Kansas City, which is a facility accredited by the American Association for the Accreditation of Laboratory Animal Care. Before and after the surgical procedures, rats were maintained in the Animal Care Facility of the University of Missouri–Kansas City and were maintained on a 12:12-hour light–dark cycle with free access to food and water. The specific experimental protocols under which this work was carried out were approved by the Institutional Animal Care and Use Committee of the University of Missouri–Kansas City.

Results

Crocetin Administration Reduced Levels of Hepatic Apoptosis

As summarized in the Methods section, rats were subjected to 1 hour of sublethal hemorrhagic shock, after which they were resuscitated with LR and shed blood either with or without crocetin at 2 mg/kg. Sham animals were not subjected to shock but received a bolus administration of either saline or crocetin. Hepatic biopsy tissues taken at 24 hours post resuscitation were then assessed for levels of tissue apoptosis. The results from these studies, summarized in Figure 2, indicate that the extent of hepatic apoptosis was significantly increased at 24 hours in the control group whereas hepatic tissue obtained from the crocetin-treated rats showed no significant increase above baseline. We conclude from this experiment that crocetin provides significant protection of experimental animals against hepatic apoptosis induced by hemorrhagic shock.

It is generally recognized that an early event in development of apoptosis is the release of cytochrome c from mitochondria. Therefore, it was postulated that levels of free cytosolic cytochrome c should be significantly decreased in hepatic tissues of rats treated with crocetin
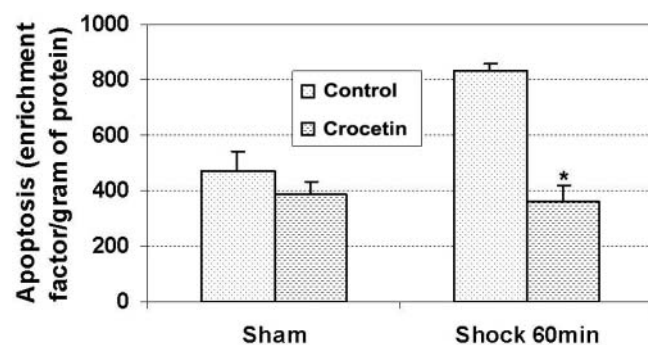
Figure 2. Acute studies. Liver cell apoptosis in sham and shock, comparing control and crocetin groups, measured at 24 hours following 60 minutes of hemorrhagic shock and 30 minutes of resuscitation or an equivalent length of time for the sham-operated animals. *$P < .05$ (control vs crocetin).
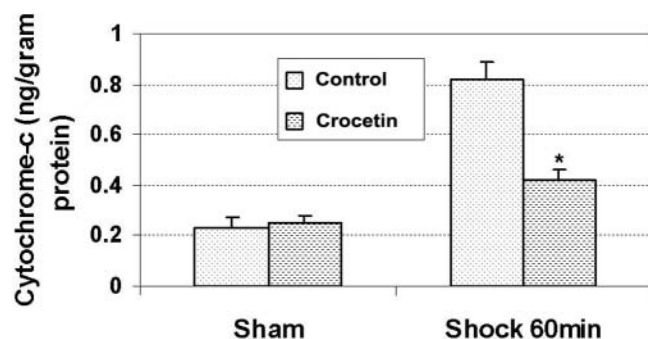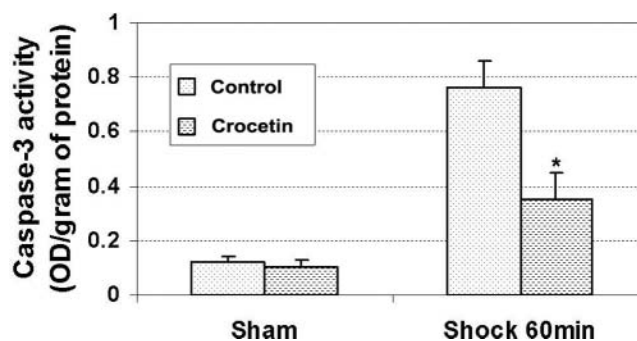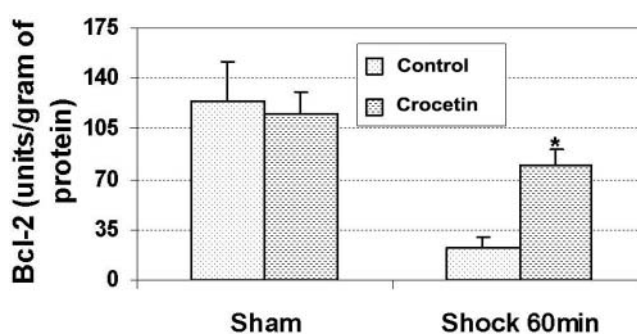

Figure 4. Acute studies. Caspase-3 activity in sham and shock, comparing control and crocetin groups, measured at 24 hours following 60 minutes of hemorrhagic shock and 30 minutes of resuscitation or an equivalent length of time for the sham-operated animals. *$P < .05$ (control vs crocetin).

Figure 3. Acute studies. Liver cytosolic cytochrome c levels in sham and shock, comparing control and crocetin groups, measured at 24 hours following 60 minutes of hemorrhagic shock and 30 minutes of resuscitation or an equivalent length of time for the sham-operated animals. *$P < .05$ (control vs crocetin).

Figure 5. Acute studies. Bcl-2 levels in sham and shock, comparing control and crocetin groups, measured at 24 hours following 60 minutes of hemorrhagic shock and 30 minutes of resuscitation or an equivalent length of time for the sham-operated animals. * $P < .05$ (control vs crocetin).

compared with rats treated with LR solution alone. We measured cytosolic extracts of tissue biopsy samples obtained from the rats at 24 hours for levels of cytosolic cytochrome c. The results of these studies, shown in Figure 3, indicate that although cytosolic cytochrome c was clearly present in both groups 24 hours post resuscitation, the levels were significantly reduced in the crocetin-treated rats compared with the control rats.

It is also well established that early events of cellular apoptosis are accompanied by increases in levels of caspase 3, an important intermediary in the pathway. Furthermore, there is a reduction in levels of bcl-2, a protein known to inhibit development of apoptosis. In support of our results with cytosolic cytochrome c, significant hepatic caspase-3 activation was observed at 24 hours in the control rats, but levels were markedly reduced in the crocetin-treated animals (Figure 4). Correspondingly, hepatic bcl-2 protein levels were diminished at 24 hours post resuscitation in both of the shock

groups, but bcl-2 levels in the crocetin-treated rats were significantly higher than in the control rats (Figure 5). We conclude from these findings that crocetin administration at the time of resuscitation significantly reduces the activation of the apoptosis pathway in hepatic tissue relative to controls.

Crocetin Improved Survival Following Hemorrhagic Shock

In a separate series of studies to assess survival following hemorrhagic shock, rats were subjected to shock at MAP of 25–30 mm Hg for 60 minutes prior to resuscitation. The MAP in the control group (n = 27) and the crocetin group (n = 26) was reduced to the same level during shock. MAP in both groups recovered to baseline within 10 minutes of beginning fluid resuscitation and actually exceeded baseline values during resuscitation (Figure 6). Animals were then closely followed for 3 days and monitored for survival.
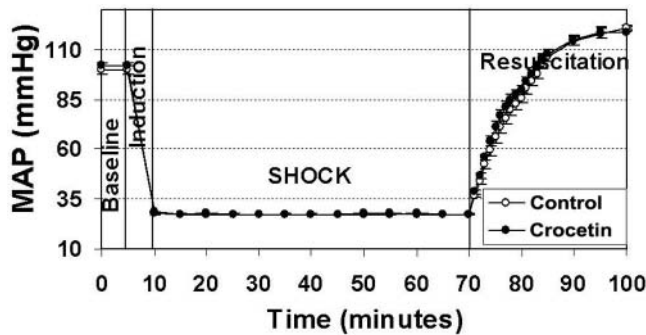
Figure 6. Chronic studies. Time course of mean arterial pressure (MAP) during 60 minutes of hemorrhagic shock followed by 30 minutes of resuscitation.
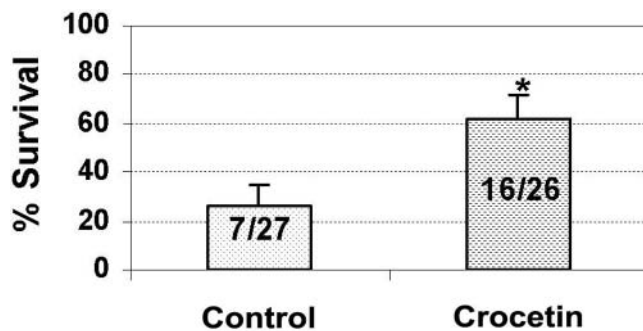

Figure 7. Chronic studies. Survival rates at 72 hours after 60 minutes of hemorrhagic shock and 30 minutes of resuscitation. *$P < .05$ (control vs crocetin).

As seen in Figure 7, administration of crocetin during resuscitation, compared with resuscitation with LR only, significantly reduced mortality following lethal hemorrhagic shock.

Discussion

Our studies provide strong evidence that inclusion of crocetin in resuscitation fluids administered to experimental animals following a period of hypovolemic shock significantly reduces mortality. One possible mechanism for this reduction is that crocetin administration may restrict the extent of cellular apoptosis that normally accompanies fluid resuscitation following hemorrhagic shock. Evidence of reduced hepatic apoptosis is provided by reductions in levels of postshock cytosolic cytochrome c, reduced levels of activated caspase 3, and increases in the levels of protective bcl-2 protein. These results collectively support the concept that administration of crocetin—or crocetin-like molecules—might be a viable treatment option for injured patients and others presenting with

shock due to hemorrhage. Mitochondrial damage, either from ischemia or from reperfusion, leads to the release of cytochrome c from the mitochondria into the cytoplasm, thereby initiating activation of the apoptotic pathway. Given that crocetin administration both protects animals from mortality following resuscitation from shock and reduces markers associated with cellular apoptosis, it is highly likely that apoptosis is a major mechanism responsible for cellular and organ damage following shock.[1,2] Specifically, we observed a marked increase in free cytochrome c detectable in the cytoplasmic fraction of hepatic tissues compared with baseline at 24 hours. With crocetin administration, however, cytosolic cytochrome c levels were inhibited significantly compared with control animals resuscitated with LR alone. Caspases are well established to be cysteine proteases that serve as key effectors in apoptotic cell death. Caspases are usually present in the cellular cytoplasm as inactive zymogens that become activated in response to a variety of specific death stimuli. The appearance of active caspase-3 is generally accepted as strong evidence that the apoptotic pathway has been activated and that a cell will proceed to apoptotic cell death.[11] Our data also provide strong evidence of a marked increase in hepatic caspase-3 at 24 hours, compared with baseline, following a period of shock. Once again, crocetin administration significantly inhibited caspase-3 levels compared with controls.

The bcl-2 protein, a constituent of the mitochondrial membrane, is thought to serve a protective function for the mitochondrion against ischemic damage. Results of previous research have helped to establish that levels of bcl-2 protein are severely depressed in experimental animals following shock and resuscitation.[12-15] As might be anticipated, given the results with cytochrome c and caspase 3, levels of bcl-2 were markedly increased at 24 hours in the crocetin-treated animals relative to controls. Thus, treatment with crocetin at the time of resuscitation appears to contribute significantly to recovery of hepatic bcl-2 levels.

Following ischemia and resuscitation, both apoptosis and necrosis can be seen. They are more closely related than previously thought and they share certain common pathways. Indeed, the term "necrapoptosis" has been coined to reflect the overlap between them.[16] Paxian et al[17] observed, as we have,[8,9] that hemorrhagic shock markedly depresses hepatic ATP. They made the additional observation that resuscitation after a brief period of shock compared with a longer period of shock was associated with more rapid recovery of ATP, better return of hepatic function, and, significantly, more apoptosis compared with necrosis. Shorter periods of shock (1 and 2 hours) were associated predominantly with postresuscitation apoptosis, whereas the longest period of shock (3 hours) was associated predominantly with necrosis. Necrosis appeared to be associated with failure of ATP levels to

recover after resuscitation. Although the models are somewhat different, the present studies, both acute and chronic, used relatively shorter periods of shock (30 and 90 minutes, respectively), within the range that produced apoptosis in the studies of Paxian et al. To the extent that crocetin administration is associated with more rapid recovery of ATP, it might be expected to diminish necrosis as well. However, this study was intended to examine the apoptotic pathway and provided no evidence on whether necrosis was affected.

This study was limited to hepatic tissue. We have confirmed that intestinal mucosa exhibit similar changes to hepatic tissue in terms of ATP and adenosine metabolites. However, these experiments were not repeated in intestinal mucosa or in other tissues. It would be unwise to generalize these findings without further experimental verification.

Our findings indicate that administration of crocetin during resuscitation reduces the extent of liver apoptosis and significantly increases survival compared with resuscitation using LR alone. These results support the validity of our hypothesis that crocetin reduces cell apoptosis and increases survival. It is likely that crocetin modifies the cellular death pathway of apoptosis and protects from cellular reperfusion injuries. Of note, this may not be the only mechanism of action for this agent.

Results from our previous experiments have provided convincing evidence that crocetin administration at the time of resuscitation from hemorrhagic shock in rats accelerates the return of cellular energy stores to normal. The mechanism of action probably involves, at least in part, prevention of mitochondrial damage.[8,9] Because crocetin is highly hydrophobic, this could occur through crocetin-mediated stabilization of the mitochondrial membrane. It is also possible, as others have hypothesized, that crocetin increases oxygen transport in the plasma and may thereby enhance oxygen supply at the cellular level.[3-7] Other investigators have found that crocetin can function as an anti-inflammatory agent or as an antioxidant[18-21] and can protect against hepatotoxic agents.[20,22] Crocin, a closely related molecule, has been shown to protect against cell death in retinal photoreceptor cells.[23]

The precise mechanism or mechanisms by which crocetin exerts its protective effects remain to be fully elucidated. Given that other lipids, such as leukotriene B4, can serve as relatively potent activators of the peroxisome proliferator–activated receptor-α, which has well-established functions in inhibiting inflammatory responses, it is attractive to consider a similar mode of action for crocetin. Alternatively, others have published results that strongly implicate a role for inducible nitric oxide synthase in the hemorrhage-induced increase in caspase-3 activity.[24-26] We noted previously that crocetin administration following shock inhibits messenger RNA for inducible nitric oxide synthase.[10] Our continuing research efforts are directed toward further understanding these mechanisms, with the ultimate goal of developing effective strategies to treat hemorrhagic shock.

Conclusion

Crocetin has potential for treatment of hemorrhagic shock. The development of new pharmacologic strategies to minimize late tissue and organ damage can be expected to greatly improve the clinical treatment of hemorrhagic shock. Subsequent investigations will be designed to determine the specific underlying mechanisms and whether action is primary or secondary to increased oxygen transport.

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Appendix 3 Summary of Cell Culture Studies with Arginine

Influence of Arginine upon Hypoxia-Induced Changes in Proteasomal Structure and Function in RAW 264.7 Cells

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ABSTRACT

Introduction: Exposure of cells to hypoxia can induce an inflammatory response. Production of inflammatory mediators by RAW 264.7 cells in response to lipopolysaccharide (LPS) stimulation has long been used as a model for the response of macrophages to inflammatory stimuli. Previous work by our group and others has demonstrated that LPS stimulation of macrophage-like cells produces inflammatory mediators and changes in the proteolytic activities of the proteasome. Changes are also seen both in proteolytic protein subunits of the proteasome and in corresponding gene expressions.   Ongoing work from our laboratory has indicated that exposing cells to hypoxia produces changes in the proteasome that parallel those seen following LPS stimulation.   Other preliminary studies suggest that arginine can markedly alter the immune response both in vitro and in vivo. The hypothesis of the present work is that hypoxia-induced changes in the proteasome can be modulated by arginine.

Materials and Methods: RAW 264.7 cells were cultured in DMEM medium and exposed to an atmosphere of 1.5% oxygen and 5% CO_2 for periods up to 48 hours. Control cells were kept in 21% oxygen and 5% CO_2, The three types of proteasomal activity chymotrypsin-like, trypsin-like, and post-glutamate, were measured with chemiluminescence. The protein subunits X, Y, and Z, together with their alternates LMP7, LMP2, and LMP10, were assessed measuring both protein (Western blot) and mRNA (reverse transcription and blotting) and protein levels. ATP content was measured with a chemiluminescence assay. Cell counts and growth of cells were measured by two different ways, automated cell count after trypsin digestion and total nucleic acid analysis. Cellular ATP content was calculated from total ATP and cell counts. To assess inflammatory mediators, studies were done for TNFα, IL1β, and iNOS, assessing both protein (ELISA) and mRNA (reverse transcription and blotting) and protein levels (Western blot). Nitrite levels were measured as an index of nitric oxide synthase (NOS) activity. To determine the effect of arginine, DMEM solution with varying levels of arginine (10 to 500 micromolar) were prepared from arginine-free DMEM and cell culture grade L-arginine.

Results: Cells grew well in hypoxia at 1.5%. Growth was about 15% less than normoxic control cclls, measured either by cell count or nucleic acid analysis. The ATP content of the cells was the same for hypoxic and normoxic cells. Proteasomal activities in the hypoxic cells showed, as compared with controls, a twofold increase in chymotrypsin-like activity, unchanged trypsin-like activity, and somewhat decreased (15%) post-glutamate activity. These changes were accompanied by up-regulation of mRNA for the subunits LMP-7 and LMP-2, and appearance of the corresponding

proteins. Cytokine secretion and mRNA for cytokines were less evident. TNFα, IL-1β, and IL-6 were not stimulated by hypoxia, while iNOS mRNA and protein level were increased significantly.

By varying the arginine concentration of the culture medium, we established that the observed proteasomal changes summarized above were strongly arginine dependent. We observed a dose-dependent over-expression of inducible trypsin-like LMP-2 protease constituent of the proteasome in response to changes in arginine,

Discussion: These results support the hypothesis that hypoxia *per se* induces an inflammatory response, and that that response involves changes in the proteasome, both in structure and in function. The data suggest that the proteasomal changes can occur even with a relatively minimal cytokine response. These results also support the hypothesis that arginine can modulate hypoxia-induced changes in the proteasome thereby helping to maintain cellular homeostasis.

Original Communications

Administration of Glutamine After Hemorrhagic Shock Restores Cellular Energy, Reduces Cell Apoptosis and Damage, and Increases Survival

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ABSTRACT. *Background:* Hemorrhagic shock causes a rapid depletion of adenosine triphosphate (ATP) and an increase of the terminal metabolite xanthine. Free radicals generated from xanthine oxidase play a major role in cell injury. Programmed cell death, apoptosis, is a major pathway causing reperfusion injury. During apoptosis, cytosolic cytochrome-c is released from damaged mitochondria, and it further initiates activation of apoptosis as evidenced by the appearance of caspase-3. The bcl-2 protein serves as an antiapoptosis found on the mitochondrial membrane. Glutamine has been known as a conditionally essential nutrient and seems to have beneficial effects in critically ill patients. The hypothesis of the present study is that glutamine administered during resuscitation following hemorrhagic shock would restore the depletion of hepatic ATP, reduce cellular apoptosis, and increase survival. *Methods:* Male Sprague-Dawley rats were randomly assigned to 3 groups for resuscitation after the same pattern of hemorrhagic shock: Ringer's lactate (LR 21 ml/kg); Alanine-glycine (LR with alanine 0.15 gm/kg and glycine 0.18 gm/kg); and glutamine (LR with glutamine 0.3 gm/kg). Hepatic ATP and xanthine was measured at different time periods. Hepatic apoptosis was measured and the levels of cytosolic cytochrome-c, caspase-3 and bcl-2 were analyzed. Another group of rats were used for survival study. *Results:* Glutamine administered during resuscitation following hemorrhagic shock partially restored the depletion of hepatic ATP, reduced cellular apoptosis, and increased survival. *Conclusions:* Glutamine administration during resuscitation significantly protected the liver from tissue damage caused by hemorrhagic shock. Glutamine supplementation may offer opportunities for therapeutic intervention during and after shock. (*Journal of Parenteral and Enteral Nutrition* **31:**94–100, 2007)

Hemorrhagic shock has a profound effect on cellular energy stores. Previous studies have shown that there is a severe decrease in cellular adenosine triphosphate (ATP) levels after hemorrhagic shock.[1–4] In particular, decreased hepatic ATP levels after hemorrhage have been correlated with intracellular edema and mitochondrial damage, which lead to cell injury and cell death.[5–8]

Glutamine is a precursor of ATP. Although it can be synthesized in the body, it is depleted in stress and is thus considered to be a conditionally essential nutrient. The present study was undertaken to determine if provision of exogenous glutamine would facilitate the resynthesis and recovery of ATP. A sublethal hemorrhagic shock rat model was used. Glutamine was administrated during resuscitation. Alanine and glycine, an isocaloric mixture of amino acids, was provided as an amino acid control. The hypothesis was that glutamine, administrated during resuscitation after hemorrhagic shock, would restore the depletion of hepatic ATP, reduce cellular apoptosis, and increase survival. Hepatic ATP and its metabolite xanthine were measured. Apoptosis, cytosolic cytochrome-c, caspase 3, and bcl-2 protein were assayed. Furthermore, a survival study was performed to compare resuscitation with or without glutamine.

MATERIALS AND METHODS

Hemorrhagic Shock Model

Animals were maintained in the Animal Care Facility of the University of Missouri–Kansas City. Animals were kept on 12:12-hour light-dark cycle with free access to food and water. Male Sprague-Dawley rats (300–350 g) were randomly assigned to 3 groups (Ringer's lactate [LR], alanine-glycine and glutamine), each of which was subjected to hemorrhagic shock in a similar manner. Resuscitation was with LR and return of shed blood. In the LR group, rats received LR 21 mL/kg, with no other pharmacologic treatment. In the alanine-glycine group, rats received LR 21 mL/kg, with alanine 0.15 g/kg and glycine 0.18 g/kg. In the glutamine group, rats received LR 21 mL/kg, with glutamine 0.3 g/kg.

All rats were anesthetized with isoflurane (Baxter Healthcare Corporation, Deerfield, IL) using an anesthesia vaporizer (SurgiVet, Inc. Waukesha, WI) with 100% oxygen while breathing spontaneously. Isoflu-

rane was adjusted from 3% to 1% after induction and then maintained at 1% until the conclusion of the experiment.

Rats were covered with a surgery drape. The temperature under the drape was controlled by a lamp over the table, maintaining it approximately at 28°C during shock and 36°C during resuscitation. Vascular access was obtained over the left neck incision. Dissection was carried down to the carotid artery and jugular vein, and both were cannulated using PE50 polyethylene tubing (Becton Dickinson and Company, Sparks, MD). All animals were anticoagulated with heparin (300 units/kg) through the venous cannula after the procedure was completed. A digital blood pressure analyzer (Micro-Med, Louisville, KY) was used to monitor pressure continuously. Hemorrhagic shock was produced by withdrawing blood through the left carotid arterial cannula over a 5-minute period, until the mean arterial pressure (MAP) stabilized at 30–35 mm Hg. The MAP was maintained at that level, with further withdrawals of blood as required for 30 minutes. MAP was monitored continuously and recorded every 5 minutes.

Resuscitation was carried out by giving LR (21 mL/kg), with or without alanine-glycine or glutamine dissolved in LR, as outlined above, followed by return of the shed blood. Resuscitation fluid and blood were given slowly over a total of 30 minutes.

Liver biopsies by freeze-clamping were carried out through a small midline laparotomy incision at baseline, at the end of the shock period, and 30 minutes and 60 minutes after completion of resuscitation.[2] Separate groups of 7–8 animals were harvested at 24 and 48 hours after the shock period. In these animals, no laparotomy was done initially. After resuscitation, they were decannulated and allowed to recover. At the later time, general anesthesia was induced, a midline laparotomy incision was made, liver biopsies were taken, and the animals were killed. All liver samples were frozen in liquid nitrogen immediately and then stored at −80°C until further studies were done.

High-Performance Liquid Chromatography

Separation of ATP and its metabolites, including xanthine, was carried out using gradient high-performance liquid chromatography (Beckman Instruments, Fullerton, CA) using a Supelcosil LC-18-T reverse-phase analytical column (Supelco, Bellefonte, PA). Integration of peak areas was performed on the HP3392A electronic integrator (Hewlett-Packard, Monterey, CA). The Beckman Gold System software was used for both system control, output display, and data processing.[2,4]

Apoptosis

Liver cytosolic extract was analyzed for apoptosis, cytochrome-c, caspase-3, and bcl-2 by using an enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's recommendation. Apoptosis was assayed by using a Cell Death Detection ELISA-PLUS kit (Roche Applied Science, Indianapolis, IN). Cytochrome-c was analyzed by using an assay kit (MBL

International, Woburn, MA). Caspase-3 was assayed by using a Caspase-3 Protease Assay kit (BioSource International, Inc, Camarillo, CA). Bcl-2 was analyzed by using a bcl-2 ELISA kit (Oncogene Research Products, San Diego, CA).

Briefly, standards or experimental samples were added to microplates coated with the monoclonal antibody of interest, followed by the addition of a biotinylated second antibody. After the first incubation and washing, streptavidin-peroxidase (enzyme) was added and conjugated. After second incubation and washing, substrate solution was added and incubated. The determination was assessed by absorbance at 450 nm using a microtiter plate reader (Dynatech Laboratories, Chantilly, VA).

Survival

For survival studies, separate groups of rats were subjected to hemorrhagic shock at 30 or 90 minutes, followed by resuscitation with LR (21 mL/kg), with or without glutamine (0.3 g/kg) dissolved in LR, plus return of shed blood. Animals were monitored for 72 hours.

Statistical Analysis

Data were expressed as mean ± SEM and were analyzed using Microsoft Excel (Microsoft Corp, Redmond, WA). Data were further analyzed by using Kruskal-Wallis multiple-comparison z-value test (NCSS, Kaysville, UT). One-tail analysis of variance was used to compare survival rates (Microsoft Excel). Data were further analyzed with Fisher's exact test (http://www.matforsk.no/ola/fisher.htm.). The significance level was .05 for all analyses.

Institutional Approval

All animal care and experimental procedures were carried out according to the Guidelines of the Laboratory Animal Center of the University of Missouri–Kansas City, which is accredited by the American Association for the Accreditation of Laboratory Animal Care. The experimental protocols under which this work was carried out were approved by the Institutional Animal Care and Use Committee of the University of Missouri–Kansas City.

RESULTS

Glutamine Restores Cellular Energy After Hemorrhagic Shock

The baseline values of ATP and xanthine were similar in the different groups before the initiation of shock. Compared with the baseline, as can be seen in Figure 1, hepatic ATP levels were significantly reduced after shock. The groups receiving LR alone and LR plus alanine-glycine both showed ATP depletion at 30 and 60 minutes postresuscitation, and ATP levels remained depressed at 24 hours. Hepatic ATP levels in the group that received glutamine rose toward baseline levels more quickly at 30 minutes postresuscitation, and by 60 minutes they were not significantly different
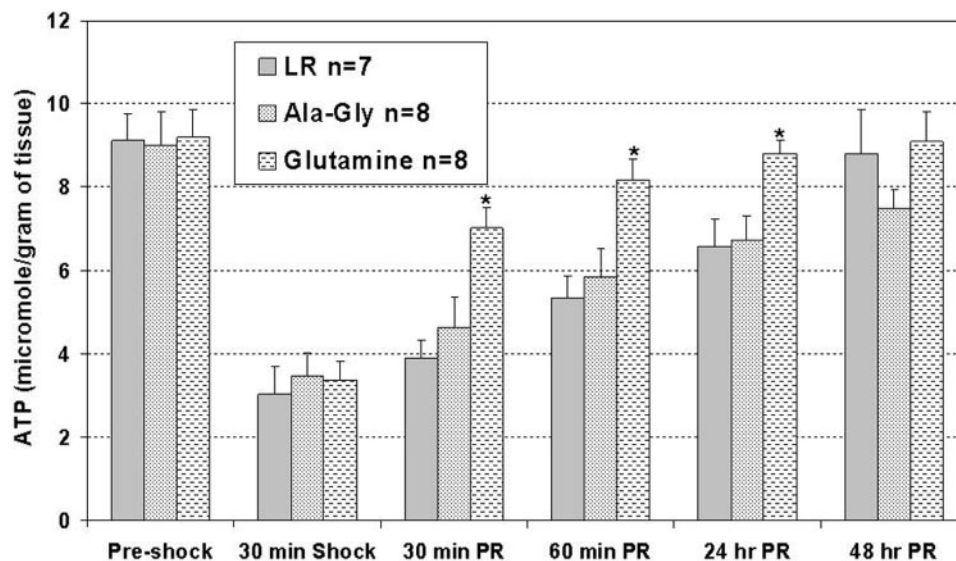
FIGURE 1. Hepatic adenosine triphosphate (ATP) concentrations in 3 groups of animals, measured at time intervals before shock, after 30 minutes of shock, and postresuscitation (30 minutes, 60 minutes, 24 hours, and 48 hours; PR). * *p*<.05 (Glutamine *vs* LR [Ringer's lactate] and Ala-Gly [alanine-glycine]).

from baseline levels. Xanthine levels were significantly increased after shock in all groups (Figure 2). At 60 minutes postresuscitation, hepatic xanthine levels were lower in the glutamine group than in the LR and alanine-glycine groups.

Glutamine Administration Diminishes Tissue Apoptosis

Apoptosis was increased at 24 and 48 hours in the LR and alanine-glycine groups, whereas the glutamine group showed no significant increase above baseline (Figure 3).

Cytosolic cytochrome-c was present 24 and 48 hours postresuscitation. The level was significantly less in the glutamine groups compared with the LR and alanine-glycine groups (Figure 4).

Hepatic caspase-3 activation was observed at 24 and 48 hours in the LR and alanine-glycine groups. It was significantly less in the glutamine group (Figure 5).

Liver bcl-2 protein was diminished at 24 and 48 hours postresuscitation. However, bcl-2 levels in the glutamine group was significantly higher than in the 2 control groups (Figure 6).

Glutamine Improves Survival After Hemorrhagic Shock

In separate studies of survival, the animal preparation was modified so that either a 30-minute or a 90-minute period of shock was used before resuscitation. Animals were followed for 3 days. Administration of glutamine during resuscitation enhanced survival
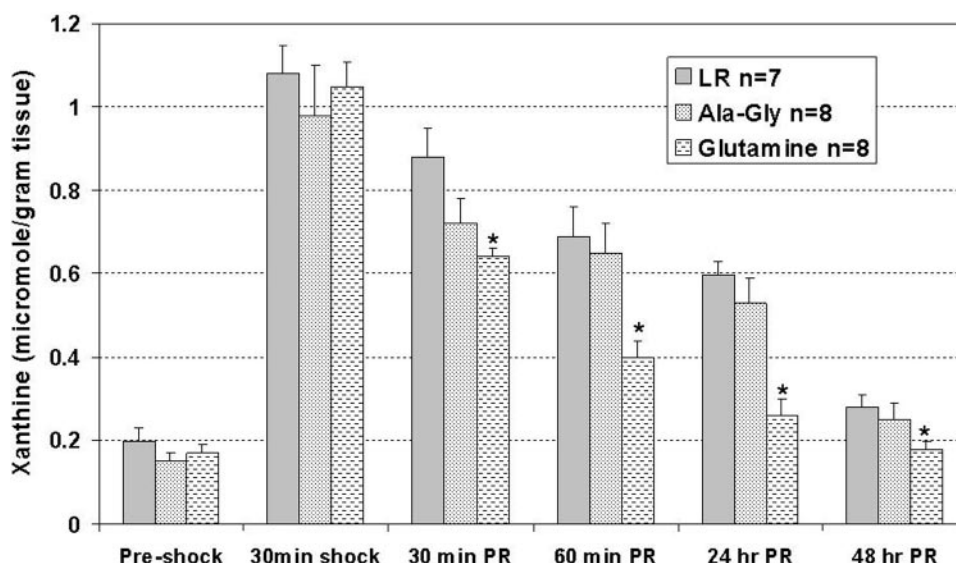

FIGURE 2. Hepatic xanthine concentrations in 3 groups of animals, measured at time intervals before shock, after 30 minutes of shock, and after resuscitation (30 minutes, 60 minutes, 24 hours, and 48 hours; PR). * *p*<.05 (glutamine *vs* LR and Ala-Gly).
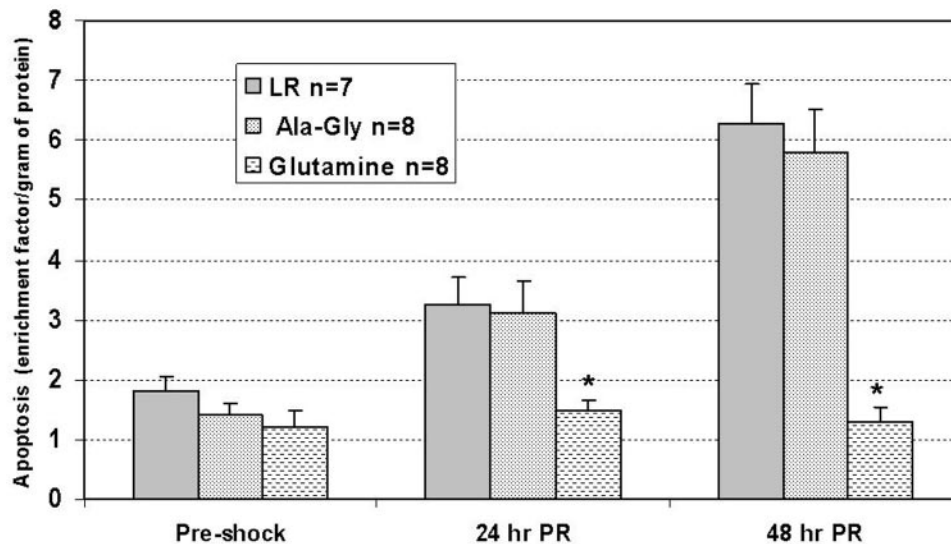
FIGURE 3. Liver cell apoptosis in 3 groups of animals, measured at preshock and postresuscitation (PR) 24 and 48 hours. * $p<.05$ (Glutamine *vs* LR and Ala-gly).

after the 90-minute shock period significantly compared with resuscitation with LR alone (Figure 7).

DISCUSSION

The data presented support the hypothesis that glutamine, when administered during resuscitation after hemorrhagic shock, will restore hepatic ATP. Work in our laboratory has studied the effect of shock on cellular nucleotide levels.[9] During normal metabolism, ATP is metabolized to adenosine, inosine, and finally to hypoxanthine and xanthine.[2,9] Hemorrhagic shock has been shown to cause depletion of ATP and corresponding elevation of all ATP metabolites.[9–11] Xanthine accumulates during ischemia. Then, when oxygenation is restored, the action of xanthine oxidase on xanthine causes a burst of oxygen free radicals, which in turn lead to cause cellular injury.[12–15] ATP levels are reconstituted relatively slowly after even a brief period of shock, returning to baseline only after 24–48 hours, whereas elevated levels of xanthine may persist beyond that time.[2,4,7,12]

In the present study, all groups of animals showed the expected reduction in liver ATP and increase in xanthine after 30 minutes of hemorrhagic shock. In the groups receiving LR and alanine-glycine, these metabolic alterations persisted, for up to 48 hours postresuscitation. In contrast, animals treated with glutamine during resuscitation showed return of ATP tissue levels to preshock levels in about 2 hours. Some elevation of xanthine was seen even at 48 hours, but it was less than that seen in the control groups.
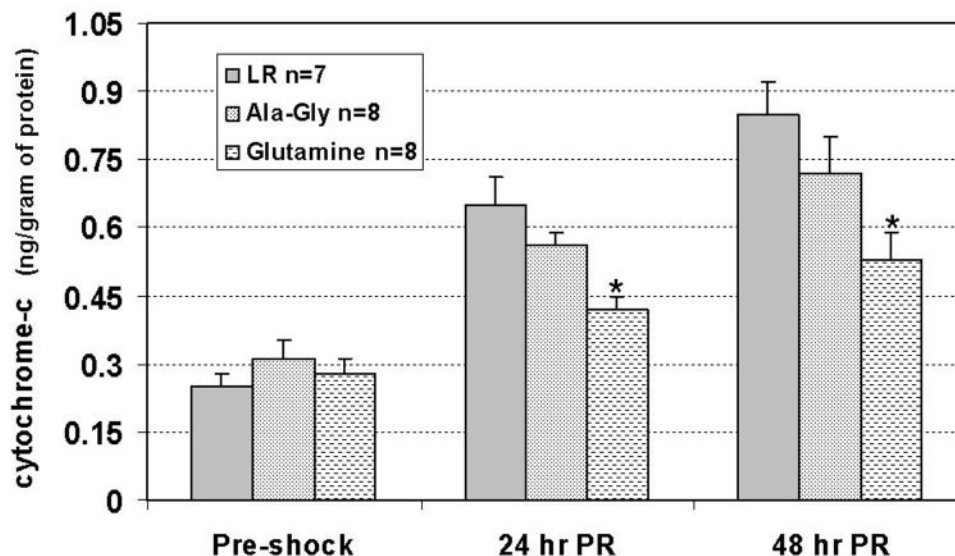

FIGURE 4. Liver cytosolic cytochrome-c levels in 3 groups of animals, measured at preshock and postresuscitation (PR) 24 and 48 hours. * $p<.05$ (Glutamine *vs* LR and Ala-Gly).
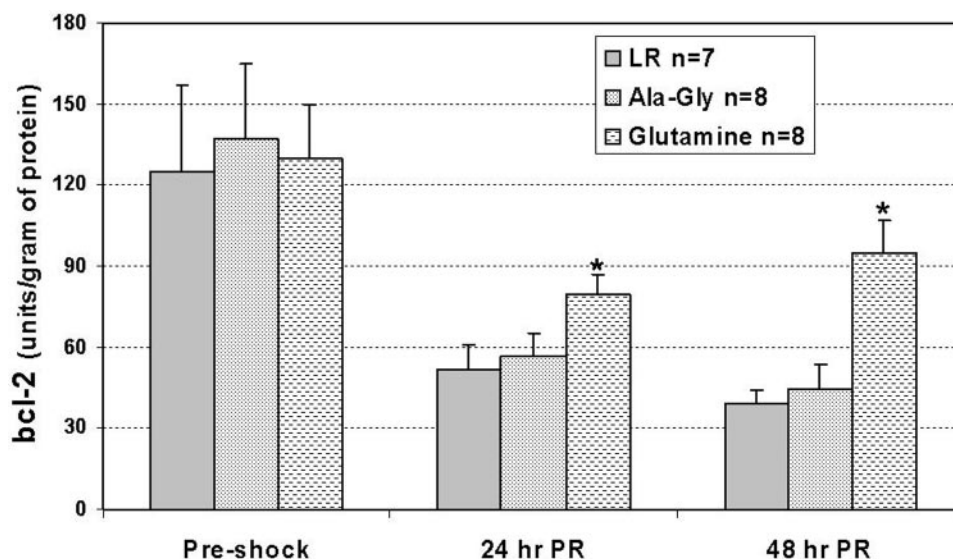
FIGURE 5. Bcl-2 activity in 3 groups of animals, measured at preshock and postresuscitation (PR) 24 and 48 hours. * $p < .05$ (Glutamine *vs* LR and Ala-Gly).

It is highly likely that apoptosis is a major mechanism by which organ damage results from reperfusion injury.[16,17] Apoptosis is initiated by mitochondrial damage. Cytochrome-c is released into the cellular cytoplasm from damaged mitochondria. Its release is a signal to initiate activation of the apoptotic pathway. As shown in Figure 4, there was a marked increase in free cytochrome-c detectable in the cytoplasmic fraction of hepatic tissues compared with baseline at 24 and 48 hours in the LR and alanine-glycine groups. In the glutamine group, by contrast, cytosolic cytochrome-c levels remained near baseline.

The appearance of caspase-3 is generally accepted as evidence that the apoptotic pathway has been activated and will proceed to apoptotic cell death.[18] As shown in Figure 5, there was a marked increase in hepatic caspase-3 compared with baseline at 24 and 48 hours after a period of shock in the LR and alanine-glycine groups. In the glutamine group, caspase-3 levels remained near baseline.

The bcl-2 protein, a constituent of the mitochondrial membrane, is thought to serve a protective function for the mitochondrion against ischemic damage. Previous research has established that levels of bcl-2 protein are severely depressed after shock and resuscitation.[19–21] As shown in Figure 6, levels of bcl-2 were markedly depressed at 24 and 48 hours in the LR and alanine-glycine groups but significantly less so in the glutamine group. Treatment with glutamine appeared to contribute to recovery of hepatic bcl-2 levels after shock.

Ultimately, any effective therapeutic agent must improve survival. Importantly, the administration of glutamine during resuscitation significantly enhanced survival in this shock model.
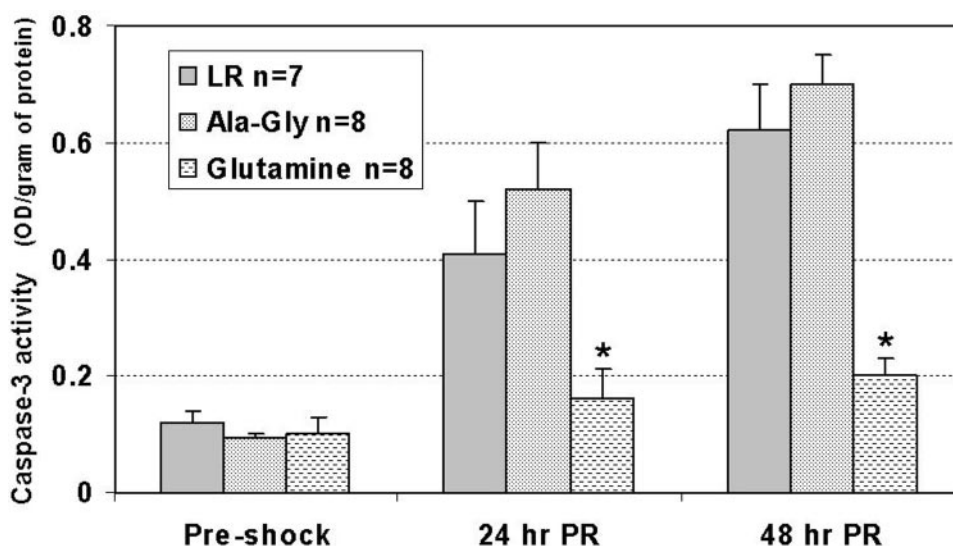

FIGURE 6. Caspase-3 levels in 3 groups of animals, measured preshock and postresuscitation (PR) 24 and 48 hours. * $p < .05$ (Glutamine *vs* LR and Ala-Gly).
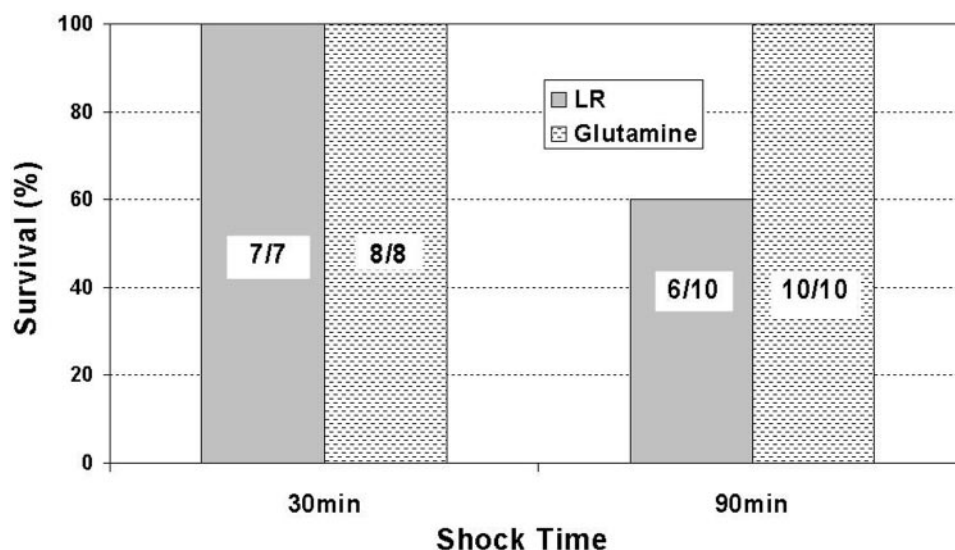
FIGURE 7. Survival rates at 72 hours after shock periods of 30 minutes and 90 minutes compares Ringer's lactate (LR) alone to LR plus glutamine. * $p<.05$ (Glutamine *vs* LR).

In summary, glutamine is a precursor of ATP synthesis. In stress states, it may be present in the body in insufficient quantities. During conditions of stress, glutamine levels have been documented to decrease dramatically in blood and extracellular fluid, and these conditions are reported to persist throughout recovery.[22,23] Glutamine supplementation has been advocated for patients under stress caused by infection, recent surgery, or traumatic injury.[24–26] Furthermore, glutamine appears to be an immunomodulator and has been used in immune-enhancing nutrition formulas.[27,28] Work from our laboratory has shown that administration of the alanine-glutamine dipeptide, a glutamine source, can suppress messenger RNA (ribonucleic acid) for the cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β, and for inducible NO synthetase (iNOS).[29] The mechanism by which glutamine exerts these effects is not understood, although there is recent published evidence that it may inhibit NFκB activation through suppression of ubiquitination of IκB, thus preventing its degradation by the proteasome.[30–33] Ziegler et al[34] and Wischmeyer et al[35] have postulated that it exerts a protective effect by stimulating the production of heat shock protein (HSP)-70.

CONCLUSIONS

Glutamine supplementation significantly protected the liver from tissue damage caused by hemorrhagic shock. Glutamine administration during resuscitation is associated with more rapid return of cellular energy stores toward normal levels. Agents such as glutamine seem to favorably modulate the inflammatory response to shock and resuscitation and prevent cellular damage. Use of such agents may diminish the postshock systemic inflammatory reaction and thus lower the risk of organ failure and subsequent death. Glutamine supplementation may help to protect cellular energy stores in the stressed organism and may offer opportunities for therapeutic intervention during and after stress.

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